이학박사학위논문

DNA 손상시 유도되는 DBC1의 수모화가 p53에 의해 매개되는 세포사멸에 미치는 영향에 관한 연구

Studies on the effect of DBC1 modification by SUMO2/3 on p53-mediated apoptosis in response to DNA damage

2016년 8월

서울대학교 대학원

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지도교수 정 진 하

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By
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ABSTRACT

Studies on the effect of DBC1 modification by SUMO2/3 on p53-mediated apoptosis in response to DNA damage

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SIRT1, a mammalian ortholog of yeast silent interaction regulator 2 (Sir2), is a NAD^+-dependent histone III deacetylase. SIRT1 regulates various cellular processes, such as apoptosis, stress response, tumorigenesis, and metabolism. Tumor suppressor p53 is a main target for SIRT1. Under normal conditions, p53 is deacetylated by SIRT1, inactivated, and degraded by MDM2, the major ubiquitin E3 ligase. Under stress conditions (e.g., exposure to UV or etoposide), however, p53 is acetylated by p300/CBP, dissociated from MDM2 for stabilization, and activated, resulting in p53-mediated induction of cell cycle arrest or apoptosis.

Small ubiquitin-related modifier (SUMO) is an ubiquitin-like protein that is conjugated to a variety of cellular proteins. Like ubiquitin, SUMO is conjugated to target proteins by a three enzyme cascade system consisting of SUMO-activating E1 enzyme (SAE1/SAE2), SUMO-conjugating E2 enzyme (Ubc9), and SUMO E3 ligases (PIASs). Conjugated SUMO can be removed by a family of Sentrin-specific proteases (SENPs). This reversible sumoylation process regulates diverse cellular processes, including transcription, nuclear transport, stability, and signal transduction.

Deleted in breast cancer 1 (DBC1) is a tumor suppressor that plays crucial roles in the control of diverse cellular processes, including stress response and energy metabolism. DBC1 is a major inhibitor of SIRT1. Under DNA damage conditions, DBC1 binds to SIRT1 and this tight binding displaces p53 from SIRT1, allowing acetylation and transactivation of p53 for expression of its downstream targets, such as p21, BAX, and PUMA. However, how the function of DBC1 is regulated remained unknown.

Phosphorylation of DBC1 regulates DBC1-SIRT1 interaction and SIRT1 deacetylase activity. Under stress conditions, ATM/ATR kinases are activated and phosphorylates DBC1 at Thr454. This phosphorylation causes tight binding between DBC1 and SIRT1, leading to dissociation of p53 from SIRT1 for subsequent acetylation and transactivation of p53.

In this study, I demonstrated that DBC1 is a target for SUMO modification and that Lys591 serves as the major SUMO acceptor site. Treatment with DNA-damaging agents, such as etoposide and doxorubicin, induced sumoylation of endogenous DBC1. In addition, DBC1 was modified by SUMO2 and SUMO3, but not by SUMO1. Remarkably, this sumoylation of DBC1 promoted its interaction with SIRT1, leading to p53 acetylation.

PIAS3 was found to act as a DBC1-specific SUMO E3 ligase and SENP1 was to serve as DBC1-specific desumoylation enzyme. Interestingly, PIAS3 and SENP1 interacted to the same N-terminal region of DBC1 and therefore competed with each other for binding to DBC1. Etoposide treatment reduced the interaction of DBC1 with

SENP1, but promoted that with PIAS3, resulting in an increase in DBC1 sumoylation. Remarkably, the switching from SENP1 to PIAS3 for DBC1 binding was achieved by ATM/ATR-mediated phosphorylation of DBC1. These results demonstrate that PIAS3 and SENP1 antagonistically regulate SUMO modification of DBC1. Consistently, SENP1 knockdown promoted etoposide-induced apoptosis, whereas knockdown of PIAS3 or SUMO2/3 and overexpression of sumoylation-deficient DBC1 mutant inhibited it. Collectively, the present findings indicate that SUMO modification of DBC1 by SUMO2/3 plays a crucial role in p53-mediated apoptosis under DNA damage conditions.

Key word: DBC1 (Deleted in brease cancer 1), p53, PIAS3 (protein inhibitor of activated STAT3), SENP1 (sentrin/sumo-specific protease 1), SUMO, phosphorylation, apoptosis

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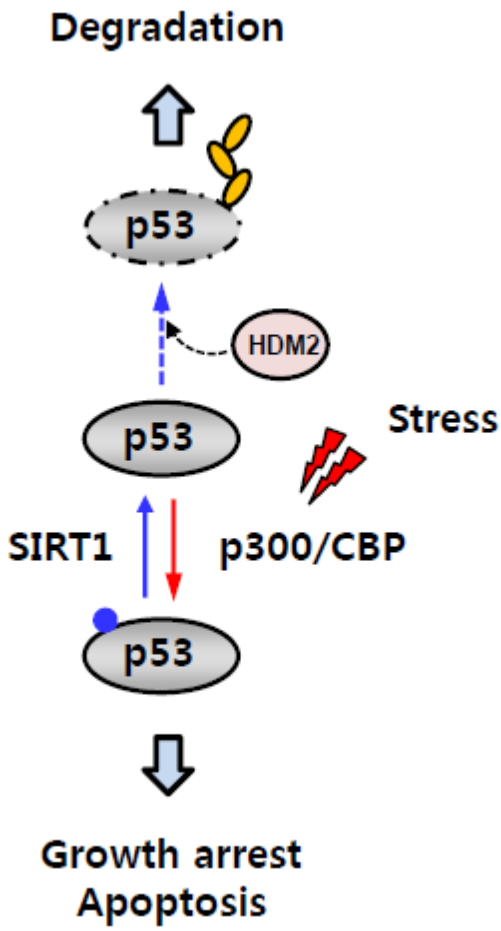
BACKGROUND

1. p53 pathway

The p53 tumor suppressor has been regarded as “cellular gatekeeper” (Lane, 1992), as it coordinates cellular responses to various stress signals, such as DNA damage, abnormal oncogene activation, telomere erosion, and hypoxia (Green & Kroemer, 2009). Under normal conditions, p53 is down-regulated by several ubiquitin E3 ligases, including the major MDM2 ligase, and subsequent degradation by proteasome. Notably, expression of MDM2 is induced by p53, thus forming a negative feedback loop for keeping p53 at a low level (Ashcroft & Vousden, 1999; Wu et al, 1993). Under stress conditions, however, p53 is stabilized and activated by disruption of its interaction with MDM2 or SIRT1 and the other negative regulators through phosphorylation by ATM/ATR kinases and acetylation by p300/CBP. The activated p53 then binds to a specific DNA sequence, called the p53-responsive element (*p53RE*), for

Figure 1. p53 pathway.

Under normal conditions, p53 is deacetylated by SIRT1, inactivated, and degraded by E3 ligase MDM2 for proteasome. Under stress conditions, DNA damage activates various regulatory enzymes that can phosphorylate and acetylate p53. Phosphorylated and acetylated p53 is then dissociated from MDM2 for stabilization. The activated p53 induces cell cycle arrest or apoptosis


Degradation
p53
HDM2
p53
Stress
SIRT1
p300/CBP
p53
Growth arrest
Apoptosis

transcriptional activation of its target genes (e.g., *CDKN1, BAX, and PUMA*) that mediate cell cycle arrest and apoptosis (Miyashita & Reed, 1995).

Since p53 is involved in the control of numerous critical cellular processes, its transactivity needs to be tightly regulated (Brooks & Gu, 2003). The p53 activity is regulated by a wide variety of post-translational modifications, including the modification by ubiquitin-like proteins, in addition to phosphorylation, methylation, acetylation, and ubiquitination. Whereas MDM2- and FBXO11-mediated neddylation inhibits p53-mediated transcriptional activation (Abida et al, 2007), sumoylation promotes it (Gostissa et al, 1999).

2. SIRT1

SIRT1, a mammalian ortholog of yeast silent information regulator 2 (Sir2), is a NAD^+-dependent deacetylase (Imai et al, 2000). SIRT1 deacetylates a variety of cellular proteins, including histones, p53, PGC1, forkhead transcription factors, NF kB, Ku70, MyoD, and PPARr, implicating its important roles in the control of diverse cellular

processes, such as gene silencing, stress response, DNA repair, heterochromatin formation, and glucose metabolism (Chalkiadaki & Guarente, 2012; Qiang et al, 2012; Schwer & Verdin, 2008). In addition, SIRT1 was shown to extend the lifespan of yeast, *Caenorhabditis elegans*, and *Drosophila* (Lin et al, 2000; Wood et al, 2004), although this effect depends on the genetic background of the organisms (Burnett et al, 2011). Given the important physiological functions, the activity of SIRT1 is regulated by multiple mechanisms, including the cellular NAD^+ level, the endogenous inhibitor nicotinamide (Bitterman et al, 2002), and post-translational modifications, such as phosphorylation and sumoylation (Kang et al, 2009). In addition, active regulator of SIRT1 (AROS) was shown to activate SIRT1 through protein-protein interaction (Kim et al, 2007).

3. Small Ubiquitin-like Modifier (SUMO)

The SUMO gene (SMT3) was initially identified in *Saccharomyces cerevisiae* in a genetic screen for suppressors of the centromeric protein Mif2. SUMO modification

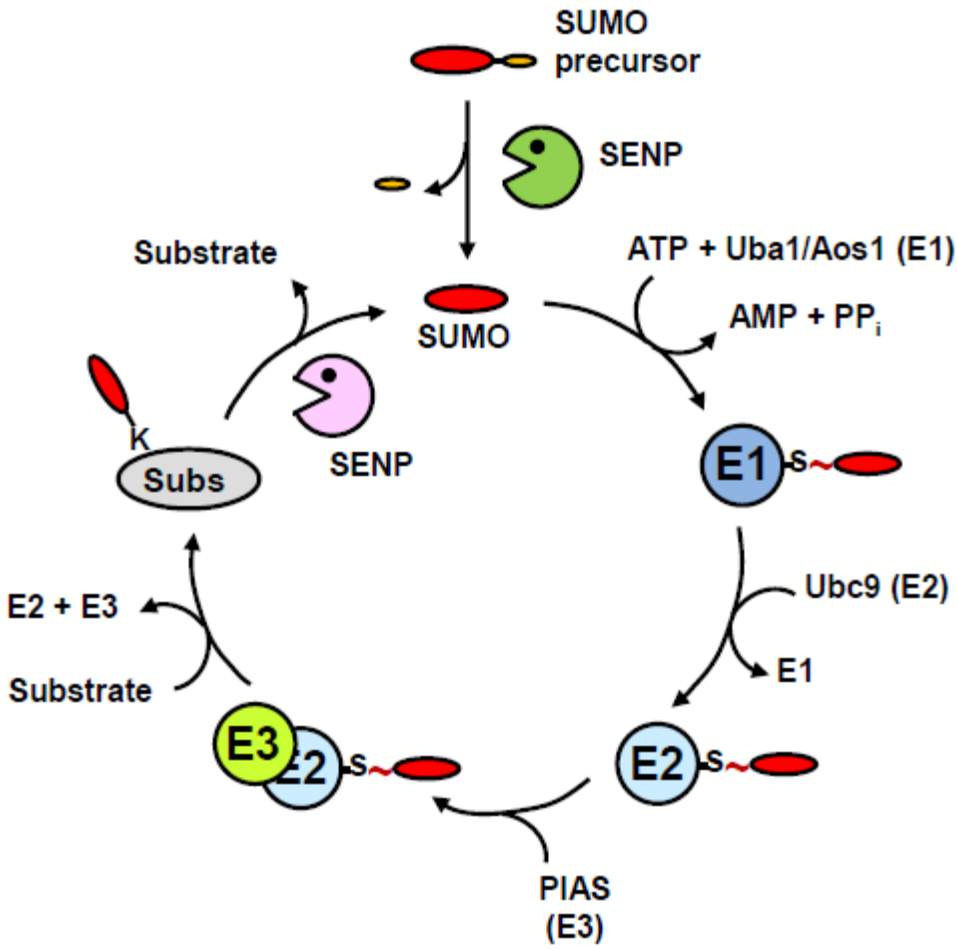
(sumoylation) leads to covalent attachment of SUMO to specific lysine residues of target proteins (Kim et al, 2002; Yeh, 2009). Amino acids sequence of SUMO is 16% identical to ubiquitin sequence, but its three-dimensional structure almost completely overlaps with that of ubiquitin. Isoforms of the SUMO family are present in yeast, plants, and metazoan. The SUMO family in metazoan consist of four related proteins, SUMO1 (also known as PIC1, Ubl1, GMP1, sentrin, Smt3c or hSmt3), SUMO2 (sentrin2 or Smt3a), SUMO3 (sentrin3 or Smt3b), and SUMO4. SUMO1-SUMO3 are ubiquitously expressed, whereas SUMO4 is expressed in the kidney, lymph node and spleen. SUMO2 and SUMO3 have 95% identical sequence, but SUMO1 shows about 50% amino acid sequence identity with SUMO2 and SUMO3. SUMOs, like ubiquitin, have the two conserved glycine residues in their C termini, which are crucial for conjugation to target proteins. SUMOs are synthesized as precursor proteins with extensions of 2-11 amino acids, which need to be cleaved off for exposing the C-terminal glycine residues.

Sumoylation of proteins, like ubiquitination, is catalyzed by a cascade enzyme

system, consisting of a hetero-dimeric SUMO-activating E1 enzyme (Uba1/Aos1), a SUMO-conjugating E2 enzyme (Ubc9), and SUMO E3 ligases (PIAS family) (Kim et al, 2002). Three proteins have been shown to have SUMO E3 ligase activity: RanBP2, the PIAS family, and the polycomb group protein Pc2. RanBP2 and the PIAS family interact with SUMO E2-conjugating enzyme Ubc9, and promote transfer of SUMO from Ubc9 to target proteins (Johnson, 2004) (Figure 2). Conjugated SUMO can be eliminated by a family of SUMO-specific proteases (SENPs). SUMO-specific proteases are cysteine proteases that possess a conserved catalytic domain characterized by the catalytic triad (histidine, aspartate and cysteine) and a conserved glutamine residue required for the formation of the oxyanion hole in the active site (Mukhopadhyay & Dasso, 2007; Yeh, 2009). Yeast has a single SUMO-like modifier, Smt3, and two Smt3-specific proteases, Ulp1 and Ulp2. The human SENPs can be divided into three families. The first family includes SENP1 and SENP2, which have broad specificity to all three mammalian SUMO1-3.

Figure 2. SUMO modification pathway.

SUMO1-3 are conjugated to target proteins by a cascade enzyme system, consisting of E1 SUMO-activating enzyme Uba1/Aos1, E2 SUMO-conjugating enzyme Ubc9, and SUMO E3 ligases, such as the PIAS family. This process can be reversed by desumoylation enzymes, SENP family.


SUMO
precursor
SENP
Substrate
ATP + Uba1/Aos1 (E1)
SUMO
AMP + PP$_i$
K
Subs
SENP
E1
S
Ubc9 (E2)
E1
E2 + E3
Substrate
E3
E2
S
S
PIAS
(E3)

The second family including SENP3 and SENP5 favors SUMO2/3 as substrates, and are enriched in the nucleolus. They are required for mitochondrial fission and fusion. The last family contains SENP6 and SENP7. These desumoylating enzymes participate in the control of diverse cellular processes, including transcription, signal transduction, target protein stability and nuclear transport (Geiss-Friedlander & Melchior, 2007; Hay, 2005).

4. DBC1

Deleted in Breast Cancer-1 (DBC1) initially cloned from a region (8p21) homozygously deleted in breast cancer. As its name implies, DBC1 functions as a tumor suppressor. It also regulates mRNA splicing with ZIRD. DBC1 has a four functional domain. The N-terminal NLS domain of DBC1 mediates its nuclear localization and the leucine zipper domain mediates its interaction with SIRT1 (Kim et al, 2008; Zhao et al, 2008) (Figure 3). DBC1 also has an EF hand domain for calcium binding and a coiled-coil domain for interaction with target proteins.

Figure 3. Domains of DBC1 protein.

NLS (nuclear localization signal) mediates to nuclear localization. Luecine zipper domain is required for interaction with SIRT1. EF hand domain regulates calcium binding and coiled-coil domain mediates protein-protein interaction.


Leu Zipper
Coiled coil
1
923
NLS
EF hand

DBC1 interacts with SIRT1, and forms a stable complex (Kim et al, 2008; Zhao et al, 2008). DBC1 overexpression in human cells represses SIRT1 activity, leading to an increase in p53 acetylation. Thus, DBC1 plays a critical role in promotion of p53-mediated apoptosis by acting as a specific inhibitor of SIRT1. DNA damage, such as etoposide, induces phosphorylation of DBC1 on Thr454 by ATM (ataxia telangiectasia-mutated) and ATR (ataxia telangiectasia and Rad3-related) kinases (Yuan et al, 2012; Zannini et al, 2012). Phosphorylated DBC1 binds to and inhibits SIRT1, resulting in dissociation of the p53-SIRT1 complex and subsequent stimulation of p53 acetylation and p53-mediated apoptosis.

5. Purpose of thesis work

p53, a major tumor suppressor, is the first nonhistone protein found to be acetylated. The lysine residues that are acetylated also serve as the sites for ubiquitination and sumoylation, indicating the critical role of these modifications in the control of p53 function as a transcription factor. Under normal conditions, p53 is deacetylated by

SIRT1 and is degraded by MDM2 E3 ligase. However, DNA damage triggers dissociation of p53 from SIRT1, leading to the acetylation, stabilization, and upregulation p53-mediated function.

DBC1, as a major inhibitor of SIRT1, plays critical roles in the control of diverse cellular processes, including stress response and energy metabolism. DNA damage induces interaction of DBC1 with SIRT1 and finally displaces p53 from SIRT1. It triggers acetylation of p53 for induction of apoptosis. DBC1 is phosphorylated at Thr454 by ATM/ATR kinases and this phosphorylation regulates interaction of DBC1 with SIRT1. However, it has been reported that phosphorylation-mimic mutant of DBC1 is insufficient in increasing DBC1-SIRT1 interaction (Zannini et al, 2012). Thus, this study aims to elucidate the mechanism how the interaction of DBC1 with SIRT1 is regulated in response to DNA damage. Notably, many proteins involved in DNA-damage response are modified by SUMO (Altmannova et al, 2010; Dou et al, 2010; Lee et al, 2006; Lee et al, 2012) 2012). Therefore, it is important to determine whether SUMO modification is involved in the control of DBC1 function. In summary, the

purpose of this study is to clarify the molecular mechanism that controls the interaction of DBC1 with SIRT1 in response to DNA damage and to determine the relationship between phosphorylation and sumoylation.

INTRODUCTION

DBC1 was initially identified as a putative tumor suppressor, since its gene was found in a region frequently deleted in breast cancers. Intriguingly, DBC1 was later found to be a negative regulator of SIRT1 (Kim et al, 2008; Zhao et al, 2008). DBC1-mediated inhibition of SIRT1 leads to an increase in p53 acetylation and thereby the p53-mediated processes, such as apoptosis. On the other hand, down-regulation of DBC1 results in SIRT1-mediated p53 deacetylation and inhibition of stress-induced apoptosis. Moreover, by using DBC1 knockout mice, DBC1 was shown to act as a major regulator of SIRT1 in vivo (Escande et al, 2010).

DBC1 inhibits SIRT1 by direct binding to its deacetylase core and disrupting its interaction with substrates (Kim et al, 2008; Zhao et al, 2008). Recently, a C-terminal region that is essential for SIRT1 activity (called ESA) was shown to interact with the catalytic core (Kang et al, 2011). Therefore, it was proposed that DBC1 competes with the ESA region for interacting with the deacetylase core, leading to inhibition of SIRT1.

DBC1 may also regulate SIRT1 activity by sensing NAD^+ through its catalytically inactive Nudix hydrolase (MutT) domain, which is known to bind NAD^+ and ADP-ribose (Anantharaman & Aravind, 2008).

The interaction between SIRT1 and DBC1 appears to be dynamically regulated. Starvation leads to a decrease in the SIRT1-DBC1 interaction, resulting in an increase in SIRT1 activity in the liver of mice, and this effect on SIRT1 activity can be revered upon feeding with high-fat diet (Escande et al, 2010). Interestingly, activation of cAMP/PKA pathway causes dissociation of the SIRT1-DBC1 complex in an AMPK-dependent fashion, suggesting that AMPK-mediated phosphorylation of SIRT1 and DBC1 may negatively regulate their binding (Nin et al, 2012). Conversely, ATM/ATR-mediated phosphorylation of DBC1 increases its interaction with SIRT1 in response to genotoxic stress, leading to promotion of p53 acetylation and cell death (Yuan et al, 2012; Zannini et al, 2012).

SUMO is an ubiquitin-like protein that is conjugated to a variety of cellular proteins. Like ubiquitin, SUMO is conjugated to target proteins by a 3-step enzyme system: E1

SUMO activating enzyme (SAE1/SAE2), E2 SUMO conjugating enzyme (Ubc9), and SUMO E3 ligases (e.g., PIASs) (Capili & Lima, 2007; Kerscher et al, 2006; Rytinki et al, 2009). Conjugated SUMO can be removed by a family of SUMO-specific proteases (SENPs) (Mukhopadhyay & Dasso, 2007; Yeh, 2009). This reversible sumoylation process participates in the control of various cellular processes, including transcription, nuclear transport, and signal transduction (Gareau & Lima, 2010; Geiss-Friedlander & Melchior, 2007; Hay, 2005; Johnson, 2004; Kim et al, 2002). Moreover, sumoylation has been implicated in the control of DNA damage response (Altmannova et al, 2010; Bergink & Jentsch, 2009; Cremona et al, 2012; Dou et al, 2010; Hoege et al, 2002; Lee et al, 2006; Polo & Jackson, 2011; Psakhye & Jentsch, 2012; Sudharsan & Azuma, 2012). For example, hnRNP-K, a coactivator of p53, is sumoylated in response to DNA damage and this modification leads to hnRNP-K stabilization and p53-mediated cell cycle arrest (Lee et al, 2012; Moumen et al, 2005).

In the present study, I demonstrated that genotoxic stress induces the modification of DBC1 at Lys591 by SUMO2/3, but not by SUMO1. I also identified PIAS3 as a

DBC1-specific SUMO E3 ligase and SENP1 as a desumoylating enzyme for DBC1. Etoposide treatment markedly decreased the interaction of DBC1 with SENP1, whereas it promoted that with PIAS3. Remarkably, the switching of the binding partners of DBC1 was achieved by ATM/ATR-mediated phosphorylation of DBC1. Moreover, DBC1 sumoylation caused a dramatic increase in the DBC1-SIRT1 interaction, which led to the release of p53 from SIRT1 for transcriptional activation. Knockdown of SENP1 promoted etoposide-induced apoptosis, whereas that of SUMO2/3 or PIAS3 and overexpression of sumoylation-deficient DBC1 mutant inhibited it. Collectively, these results indicate that DBC1 sumoylation plays a crucial role in the control of p53-mediated apoptosis under DNA damage conditions.

MATERIALS AND METHODS

1. Plasmids and shRNAs

The cDNA of DBC1 was cloned into pcDNA-HisMax and pCMV2-Flag. shRNAs were purchased from Open Biosystems. Target sequences for shRNAs are as follows: shPIAS3, 5'-GCTGTCGGTCAGACATCATTT-3'; shSENP1, 5'-CAAAGATATTC AAACTCTA-3'. shSUMO2/3, 5'-TCAATGAGGCAGATCAGATTC-3'.

2. Cell culture and transfection

HEK293T, U2OS, and HeLa cells were grown at 37°C in DMEM supplemented with 100 units/ml penicillin, 1 mg/ml streptomycin, and 10% FBS. All transfections were carried out using Metafectene reagent (Biontex) and jetPEI DNA Transfection Reagent (Polyplus-transfection).

3. Assays for SUMO modification

HisMax-DBC1, Flag-SUMO3, and Flag-Ubc9 were overexpressed in HEK293T cells with or without Myc-tagged PIAS3 or SENP1. After culturing for 40 h, cells were lysed by boiling for 10 min in 150 mM Tris-HCl (pH 8), 5% SDS, and 30% glycerol. Cell lysates were diluted 20-fold with buffer A [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1X protease inhibitor cocktail (Roche)] containing 10 mM imidazole and 2 mM NEM. After incubating them with Ni^{2+}-NTA-agarose for 2 h at 4°C, the resins were collected, washed with buffer A containing 20 mM imidazole, and boiled in SDS-sampling buffer. Supernatants were subjected to SDS-PAGE followed by immunoblot analysis. For assaying sumoylation of endogenous DBC1, HeLa cells treated with and without etoposide were lysed as above. Cell lysates were diluted 20-fold with buffer B [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, and 1X protease inhibitor cocktail] containing 0.2% Triton X-100 and 4 mM NEM. The samples were incubated with anti-DBC1 antibody for 2 h at 4°C and then with protein-A-Sepharose for the next 2 h. The resins were collected, washed with buffer B

containing 1% Triton X-100, and boiled. Supernatants were subjected to SDS–PAGE followed by immunoblot analysis.

4. Immunoprecipitation

Cell lysates were prepared in buffer B containing 1% Triton X-100 and 2 mM NEM. Cell lysates were incubated with appropriate antibodies for 2 h at 4°C and then with protein A-conjugated Sepharose for the next 1.5 h. Supernatants were subjected to SDS-PAGE followed by immunoblot analysis.

Antibodies against p53 (catalog #: DO-1), GAPDH (2D4A7), Ubc9 (N-15), DBC1 (H-2), and SENP1 (C-12) were purchased from Santa Cruz. Anti-acetyl p53 (Millipore, 04-1146), anti-DBC1 (Bethyl, A300-434A), anti-Flag M2 (Sigma-Aldrich, 1804) were also used. Anti-Xpress (1405573), anti-SUMO-1 (381900), anti-SUMO2/3 (51-9100) were purchased from Invitrogen. Anti-PIAS3 (4164S), anti-cleaved caspase-9 (7237S), and anti-cleaved PARP-1 (9541) were purchased from Cell signaling. For immunoblot analysis, all antibodies were diluted 1,000 fold in 3% BSA solution, except anti-Xpress

and Flag M2 antibodies were diluted 5,000 fold.

5. SIRT1 activity assay

The deacetylase activity of SIRT1 was determined by using a SIRT1 Activity Assay Kit (Abcam: catalog # ab156065) as recommended by the manufacturer. Briefly, cells were washed with cold PBS, lysed in buffer B containing 0.2% Triton X-100, and incubated on ice for 5 min. Cell lysates were treated with anti-SIRT1 antibody for 2 h at 4°C and then with protein A-conjugated Sepharose for the next 1.5 h. Precipitates were incubated with Fluoro-Substrate Peptide Solution, NAD^+, and SIRT1 Assay Buffer. Fluorescence intensity was then measured using a microtiter plate fluorometer with excitation at 350 nm and emission at 450 nm.

6. Determination of NAD^+/NADH ratio

Intracellular NAD^+/NADH ratio was determined by using the NAD^+/NADH Quantification Colorimetric Kit (BioVision: catalog # K337-100) as recommended by

the manufacturer. Briefly, cells were washed with ice-cold PBS, and disrupted by freezing-and-thawing. After centrifugation, one-half of the extracts were subjected to quantification of the total amount of NAD^+ plus NADH by using appropriate buffers and reagents provided by the manufacturer. The other half was heated at 60°C for 30 min to decompose NAD^+, followed by determination of the amount of heat-stable NADH. The ratio of NAD^+/NADH was then calculated by subtracting the amount of NADH from the total amount of NAD^+ plus NADH and then divided by the amount of NADH.

7. Purification of SUMO3, sumoylated DBC1, and SIRT1

To purify SUMO3, Flag-SUMO3 was expressed in HEK293T cells. Cell lysates were incubated with anti-Flag- Sepharose (Sigma) for 2 h. The beads were collected, incubated with Flag peptides (Sigma) for 30 min, and centrifuged. The supernatants were used as the purified SUMO3. To obtain sumoylated DBC1, HisMax-DBC1, Flag-SUMO3, and Myc-Ubc9 were expressed in HEK293T cells. Cell lysates were then

treated as above. The proteins eluted from the beads by Flag peptides were incubated with NTA-resins for 2 h. The resins were collected, incubated with PBS containing 200 mM imidazole for 30 min, and centrifuged. The supernatants were used as the purified SUMO3-conjugated DBC1. To purify SIRT1, GST-SIRT1 was expressed in *E. coli* (BL21). Cell extracts were incubated with glutathione- Sepharose (GE). The beads were collected, incubated with glutathione. The supernatants were used as the purified GST-SIRT1. All protein purifications were carried out at 4°C.

8. Luciferase assay

HeLa cells transfected with pcDNA-β-Gal and *PG13-Luc* or *BAX-Luc* were incubated for 48 h. After etoposide treatment, cells were cultured for 30 h, harvested, and assayed for luciferase. The enzyme activity was measured in a luminometer and normalized by β-galactosidase expression with a luciferase system (Promega).

9. TUNEL assay

After treatment with etoposide, HeLa cells were perrmeabilized by incubation in a solution containing 0.1% sodium citrate and 0.1% Triton X-100 for 30 min at 65℃. After extensive washing, samples were further incubated in the TUNEL reaction solutions of the in situ cell death detection kit (Roche Applied Science) at 37℃.

RESULTS

DBC1 is a target for sumoylation

The consensus sequence for sumoylation is Ψ-Lys-X-Glu/Asp, where Ψ is a large hydrophobic amino acid and X is any amino acid (Johnson, 2004). Upon sequence analysis, human DBC1 was found to have three potential sumoylation sites: Lys591, Lys667, and Lys839 (Figure 4). Therefore, I first examined whether DBC1 can be sumoylated upon overexpression of three SUMO isoforms. Interestingly, DBC1 was modified by SUMO2 and SUMO3, but not by SUMO1 (Figure 5). To identify the SUMO acceptor site(s) in DBC1, each of the Lys residues was substituted by Arg. The K591R mutation, but not the others, completely prevented DBC1 sumoylation (Figure 6), indicating that Lys591 serves as the major SUMO acceptor site. Since SUMO2 and SUMO3 show more than 95% identity in their amino acid sequences, I used shRNAs directed to their identical nucleotide sequence for knocking down both of them, but used only SUMO3 for overexpression.

DNA damage induces sumoylation of DBC1 and its interaction with SIRT1

Since sumoylation has been implicated in DNA damage response (Lee et al, 2012; Sacher et al, 2006), I examined whether genotoxic stress induces DBC1 sumoylation. Treatment of U2OS cells with etoposide led to a dramatic increase in SUMO2/3-modification of endogenous DBC1 (Figure 7). In contrast, little or no SUMO1-conjugated DBC1 could be detected. Moreover, knockdown of SUMO2/3 by a SUMO2/3-specific shRNA (shSUMO2/3), but not by a nonspecific shRNA (shNS), abrogated etoposide-induced DBC1 sumoylation (Figure 8), indicating that DBC1 serves as a target for modification by SUMO2/3 under genotoxic stress.

Significantly, SUMO2/3 knockdown also prevented etoposide-induced p53 acetylation (see Figure 8), raising a possibility that DBC1 sumoylation promotes the SIRT1-DBC1 interaction and in turn inhibits SIRT1-mediated deacetylation of p53. To test this possibility, I compared the abilities of DBC1 and its K591R mutant in the interaction with SIRT1. Etoposide treatment led to a dramatic increase in the interaction of SIRT1 with DBC1, but not with the K591R mutant (Figure 9). Furthermore,

knockdown of SUMO2/3 also led to a marked decrease in the DBC1-SIRT1 interaction as well as in p53 acetylation and this decrease could be reversed by complementation of shRNA-insensitive SUMO3 (Figure 10). I next examined whether the sumoylation-mediated increase in the DBC1-SIRT1 interaction can indeed inhibit the deacetylase activity of SIRT1. Overexpression of SUMO2 or SUMO3, but not SUMO1, with Ubc9 led to a marked increase in the ability of DBC1 to inhibit the SIRT1 activity (Figure 11A). Under the same conditions, however, the NAD^+/NADH ratio remained unchanged, indicating that the inhibition of the SIRT1 activity is not due to the availability of the SIRT1 substrate (Figure 11B). These results indicate that sumoylation of DBC1 is required for its interaction with and inhibition of SIRT1 and in turn for promotion of p53 acetylation under genotoxic stress.

In an attempt to determine the mechanical basis for the preferential interaction of SIRT1 with sumoylated DBC1 over its unmodified form, I examined whether SIRT1 has a SUMO-interacting motif (SIM), which consists of hydrophobic core and acidic stretch sequence franked by a spacer (Hecker et al, 2006; Song et al, 2004). Sequence

analysis showed that SIRT1 has a hydrophobic core sequence (IIVL), which is identical to that of Daxx, although it lacks an acidic stretch (Figure 12A). To determine whether the hydrophobic sequence is involved in the binding of SIRT1 to SUMO, I generated a SIRT1 mutant (KKVL), of which the first two Ile residues were replaced by Lys residues. SIRT1 bound to all of the SUMO isoforms, but this binding could be prevented by the II-to-KK mutation (Figure 12B), indicating that the hydrophobic core of SIRT1 is sufficient for interaction with SUMOs. In addition, purified SUMO3 could effectively compete with SUMO3-conjugated DBC1 for binding to SIRT1 (Figure 13). Furthermore, the II-to-KK mutation prevented the etoposide-mediated increase in the SIRT1-DBC1 interaction (Figure 14A). However, the II-to-KK mutation showed little or no effect on the ability of SIRT1 to deacetylate p53 (Figure 14B), indicating that the effect of the mutation on the SIRT1-DBC1 interaction is not mediated by misfolding or conformational change of SIRT1. These results demonstrate that the SIM-like sequence in SIRT1 is responsible for its interaction with sumoylated DBC1.

DBC1 sumoylation blocks interaction of SIRT1 with p53

To determine whether DBC1 sumoylation influences the interaction between SIRT1 and p53, HeLa cells overexpressing DBC1 and its K591R mutant were treated with etoposide. Overexpression of DBC1, but not its K591R mutant, markedly decreased the SIRT1-p53 interaction (Figure 15). Moreover, knockdown of SUMO2/3 led to an increase in the SIRT1-p53 interaction (Figure 16). Since SUMO2/3 knockdown reduced the DBC1-SIRT1 interaction (see Figure 10), it appeared that sumoylated DBC1, unlike its unmodified form, is capable of replacing p53 for its binding to SIRT1. To test this possibility, increasing amounts of SUMO3 were expressed with a fixed amount of DBC1 or its K591R mutant. The interaction of SIRT1 with DBC1 gradually increased, whereas that with p53 declined in a SUMO3 dose-dependent manner (Figure 17). In contrast, SIRT1 was unable to interact with the K591R mutant, and remained bound to p53 regardless of SUMO3 expression. These results indicate that sumoylation of DBC1 displaces p53 from SIRT1 for its binding to the deacetylase.

PIAS3 and SENP1 counteract on SUMO modification of DBC1.

To identify DBC1-specific SUMO E3 ligase, each of PIAS1-4 was overexpressed with DBC1. Among them, only PIAS3 interacted with DBC1 (Figure 18). Moreover, the DBC1-PIAS3 interaction was markedly increased by etoposide treatment (Figure 19). I next examined whether PIAS3 is indeed capable of promoting DBC1 sumoylation. Overexpression of PIAS3 dramatically increased DBC1 sumoylation (Figure 20). On the other hand, knockdown of PIAS3 by shPIAS3 prevented sumoylation of endogenous DBC1 with a marked reduction in etoposide-induced SIRT1-DBC1 interaction as well as in p53 acetylation (Figure 21). These effects of PIAS3 knockdown was confirmed by reciprocal immunoprecipitation analysis (Figure 22). Moreover, complementation of shRNA-insensitive PIAS3 reversed the effects of PIAS3 depletion on both the DBC1-SIRT1 interaction and p53 acetylation, indicating that PIAS3 serves as a SUMO2/3 E3 ligase for DBC1 and in turn as a positive regulator of p53.

To map the regions for the PIAS3-DBC1 interaction, their deletions were generated (Figure 23A and B). DBC1 bound to the N-terminal region of PIAS3 (amino acids 1-

200), and PIAS3 bound to the N-terminal region of DBC1 (1-243). In addition, the deletion of the leucine zipper motif (243-264), which is known to mediate the binding of DBC1 to SIRT1 (Kim et al, 2008), showed little or no effect on its binding to PIAS3.

To identify DBC1-specific desumoylating enzyme, each of SENP1-3 and SENP5-7 was overexpressed with DBC1. Note that SENP4, also called SUSP1, is expressed in rat and mice but not in human (Lee et al, 2006). Among the human SENPs, SENP1, SENP3, and SENP5 interacted with DBC1 (Figure 24). Without overexpression, however, only SENP1 interacted with DBC1 and this interaction was markedly reduced by etoposide treatment (Figure 25).

To determine whether SENP1 is indeed capable of desumoylating DBC1, I generated a catalytically inactive mutant of SENP1 (C603S) by substituting the active site Cys603 residue by Ser. Overexpression of SENP1, but not its C603S mutant, led to complete desumoylation of DBC1 (Figure 26). On the other hand, SENP1 knockdown caused a marked increase in DBC1 sumoylation even in the absence of etoposide and this increase was further ameliorated in its presence (Figure 27). SENP1 knockdown

also led to an increase in the DBC1-SIRT1 interaction as well as in p53 acetylation and this increase was further elevated by etoposide treatment. These effects of SENP1 depletion were confirmed by reciprocal immunoprecipitation analysis (Figure 28). Moreover, complementation of shRNA-insensitive SENP1 reversed the effects of SENP1 depletion on both the DBC1-SIRT1 interaction and p53 acetylation. These results indicate that SENP1 serves as a desumoylating enzyme for DBC1 and in turn as a negative regulator of p53.

To map the regions for the SENP1-DBC1 interaction, their deletions were generated. DBC1 bound to the N-terminal region of SENP1 (amino acids 1-200) (Figure 29A). Significantly, SENP1 bound to the N-terminal region of DBC1 (1-243) (Figure 29B), where PIAS3 also binds (See Figure 23B). Consistently, SENP1 and PIAS3 were found to compete with each other for binding to DBC1 upon analysis by expression of increasing amounts of the one over the other (Figure 30). These results indicate that DBC1 sumoylation could be dynamically regulated by competitive binding of PIAS3 and SENP1 to DBC1 under genotoxic stress.

DBC1 phosphorylation promotes its sumoylation

ATM/ATR-mediated phosphorylation of Thr454 in DBC1 was shown to increase its interaction with SIRT1 (Yuan et al, 2012; Zannini et al, 2012). To determine whether the phosphorylation of DBC1 influences its sumoylation, phosphorylation-defective (T454A) and phosphorylation-mimicking (T454D) mutants were generated by substituting Thr454 with Ala and Asp, respectively. Overexpression of the T454D mutant dramatically increased DBC1 sumoylation, whereas that of the T454A mutant decreased it (Figure 31). Moreover, the T454D mutation increased the ability of sumoylated DBC1 to bind SIRT1, whereas the T454A mutation decreased it. Furthermore, the T454D mutation promoted the DBC1-PIAS3 interaction, whereas the T454A mutation inhibited it (Figure 32). In contrast, the T454D mutation decreased the DBC1-SENP1 interaction, whereas the T454A mutation increased it. Consistently, treatment with caffeine, an ATM/ATR inhibitor, prevented the etoposide-induced association and dissociation of DBC1 with PIAS3 and SENP1, respectively (Figure 33). These results indicate that ATM/ATR-mediated DBC1 phosphorylation serves as a

switch of DBC1-binding partner from SENP1 to PIAS3 for DBC1 sumoylation, which in turn promotes the DBC1-SIRT1 interaction for p53 acetylation under genotoxic stress.

DBC1 sumoylation is required for p53 transactivation

To determine whether DBC1 sumoylation leads to transcriptional activation of p53 by sequestering SIRT1 from p53, two reporter vectors, *PG13-LUC* and *BAX-LUC*, were employed. Etoposide treatment increased the luciferase activity and this increase was further elevated by overexpression of DBC1, but not by that of the K591R mutant (Figure 34). Moreover, knockdown of SUMO2/3 or PIAS3 abrogated etoposide-induced p53 transactivity regardless of co-knockdown of DBC1 (Figure 35). In contrast, knockdown of SENP1 further increase the drug-induced p53 transactivity and this increase was abrogated by co-knockdown of DBC1 (Figure 36). These results indicate that DBC1 sumoylation is required for transcriptional activation of p53 under genotoxic stress.

DBC1 sumoylation is required for p53-mediated apoptosis

To determine whether DBC1 sumoylation-mediated p53 activation is responsible for DNA damage-induced apoptosis, I generated HeLa cells that stably express shNS or shDBC1. As expected, DBC1 knockdown prevented etoposide-mediated increase in the cleavage of PARP-1 and caspase-9 and the level of acetylated p53 (Figure 37). However, complementation of shRNA-insensitive DBC1, but not the K591 mutant, led to a significant increase in both of them in the presence of etoposide, but not in its absence. Knockdown of SENP1 also increased the cleavage of PARP-1 and caspase-9 and the level of acetylated p53 and this increase could be abrogated by co-knockdown of DBC1 (Figure 38). On the other hand, knockdown of SUMO2/3 or PIAS3 prevented both of them and these effects could be reversed by co-expression of shRNA-insensitive SUMO3 or PIAS3, respectively (Figure 39). Consistently, overexpression of DBC1, but not the K591R mutant, markedly increased the number of TUNEL-positive apoptotic cells (Figure 40). Whereas knockdown of SUMO2/3 or PIAS3 decreased the number of apoptotic cells, that of SENP1 increased the number and this increase was abrogated

by co-knockdown of DBC1 (Figure 41). These results indicate that DBC1 sumoylation is a crucial step for p53-mediated apoptosis under genotoxic stress.

I next examined whether the removal of etoposide from media could reverse the drug-induced DBC1 sumoylation and apoptosis. Withdrawal of etoposide from culture media at 48 h led to a sharp decrease in the levels of sumoylated DBC1, p53, and acetylated-p53 (Figure 42). On the other hand, the levels of cleaved PARP-1 and caspase-9 remained elevated, although did not increase any further. These results indicate that the damaged cells could not escape from apoptosis even after the removal of etoposide.

Figure 4. Consensus sequence for DBC1 sumoylation.

Three candidates sites for sumoylation are shown in bold.

ΨKXE

EAIKEEV (591)

AGAKLED (667)

LELKLEE (839)

Figure 5. DBC1 is modified by SUMO2/3, but not by SUMO1.

Flag-tagged SUMO isoforms were expressed in HEK293T cells with HisMax-DBC1 and Myc-Ubc9. Cell lysates were subjected to pull-down (PD) with NTA-resins followed by immunoblot analysis. The lysates were also directly probed with the indicated antibodies.

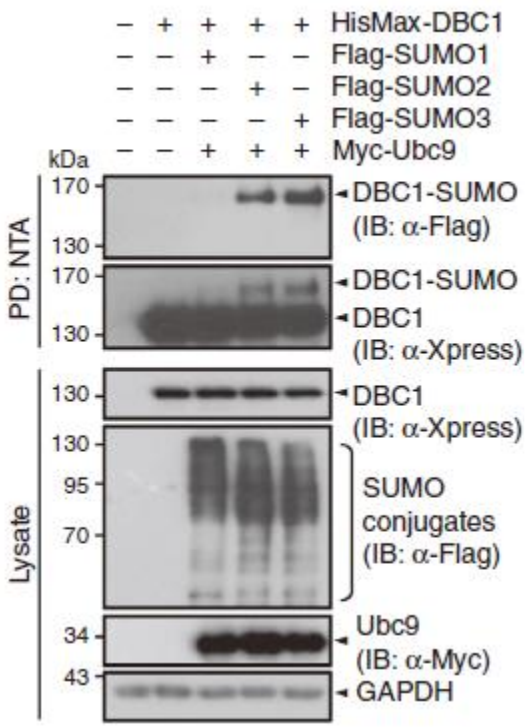

– + + + + HisMax-DBC1
– – + – – Flag-SUMO1
– – – + – Flag-SUMO2
– – – – + Flag-SUMO3
– – + + + Myc-Ubc9
kDa
PD: NTA
170
130
DBC1-SUMO
(IB: α-Flag)
170
130
DBC1-SUMO
DBC1
(IB: α-Xpress)
Lysate
130
DBC1
(IB: α-Xpress)
130
95
70
SUMO
conjugates
(IB: α-Flag)
34
Ubc9
(IB: α-Myc)
43
GAPDH

Figure 6. Lys 591 is the major SUMO acceptor site in DBC1.

HisMax-tagged DBC1 and its K-to-R mutants were expressed in HEK293T cells with Flag-SUMO3 and Flag-Ubc9. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot analysis. The lysates were also directly probed with the indicated antibodies.

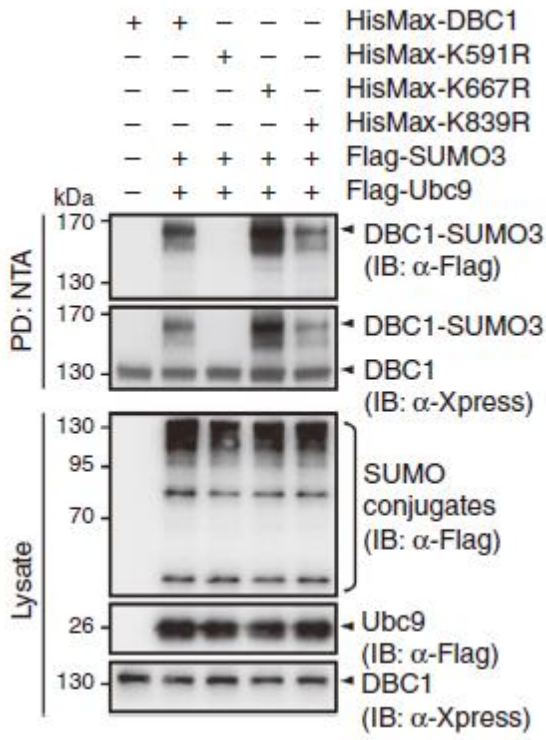

+ + – – – HisMax-DBC1
– – + – – HisMax-K591R
– – – + – HisMax-K667R
– – – – + HisMax-K839R
– + + + + Flag-SUMO3
– + + + + Flag-Ubc9
kDa
PD: NTA
170
130
DBC1-SUMO3
(IB: α-Flag)
170
130
DBC1-SUMO3
DBC1
(IB: α-Xpress)
Lysate
130
95
70
SUMO
conjugates
(IB: α-Flag)
26
Ubc9
(IB: α-Flag)
130
DBC1
(IB: α-Xpress)

Figure 7. Etoposide induces DBC1 sumoylation.

U2OS cells were treated with 20 μM etoposide (ETO) for increasing periods. Their lysates were subjected to immunoprecipitation (IP) with anti-DBC1 antibody followed by immunoblot analysis. Cell lysates were also directly probed with anti-acetylated p53 (Ac-p53), anti-p53 and anti-DBC1 antibodies.

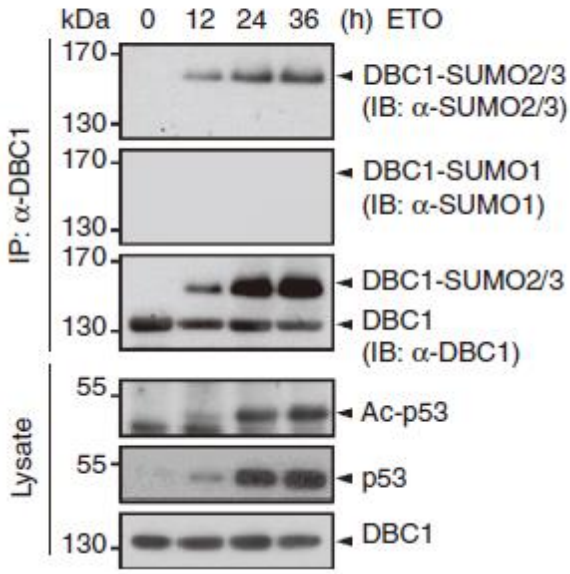

kDa 0 12 24 36 (h) ETO
IP: α-DBC1
Lysate
170
130
170
130
170
130
55
55
130
DBC1-SUMO2/3
(IB: α-SUMO2/3)
DBC1-SUMO1
(IB: α-SUMO1)
DBC1-SUMO2/3
DBC1
(IB: α-DBC1)
Ac-p53
p53
DBC1

Figure 8. Knockdown of SUMO2/3 blocks etoposide-induced DBC1 sumoylation.

U2OS cells expressing nonspecific shRNA (shNS) or shSUMO2/3 were incubated with and without etoposide for 36 h. Their lysates were then subjected to immunoprecipitation with anti-DBC1 antibody followed by immunoblot analysis.

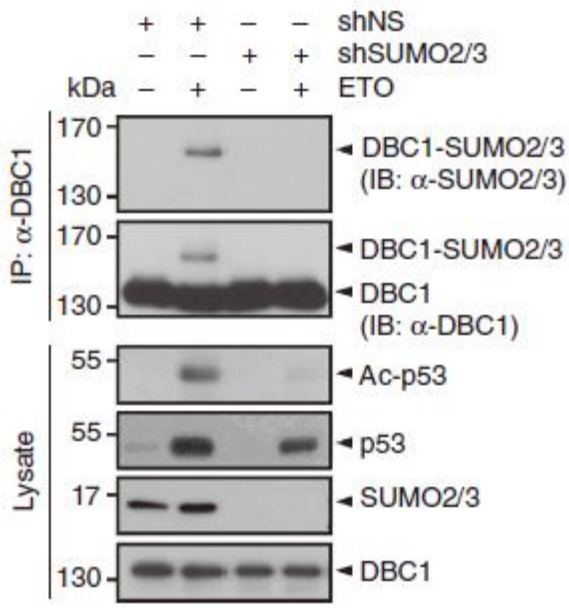

+ + − − shNS
− − + + shSUMO2/3
kDa − + − + ETO
IP: α-DBC1
170
130
DBC1-SUMO2/3
(IB: α-SUMO2/3)
170
130
DBC1-SUMO2/3
DBC1
(IB: α-DBC1)
Lysate
55
Ac-p53
55
p53
17
SUMO2/3
130
DBC1

Figure 9. Etoposide increases interaction of SIRT1 with DBC1, but not KR mutant.

HeLa cells expressing Flag-tagged DBC1 (Wt) and its K591R mutant (KR) were incubated with and without etoposide for 36 h. Their lysates were subjected to immunoprecipitation with anti-SIRT1 and anti-Flag antibodies followed by immunoblot analysis.

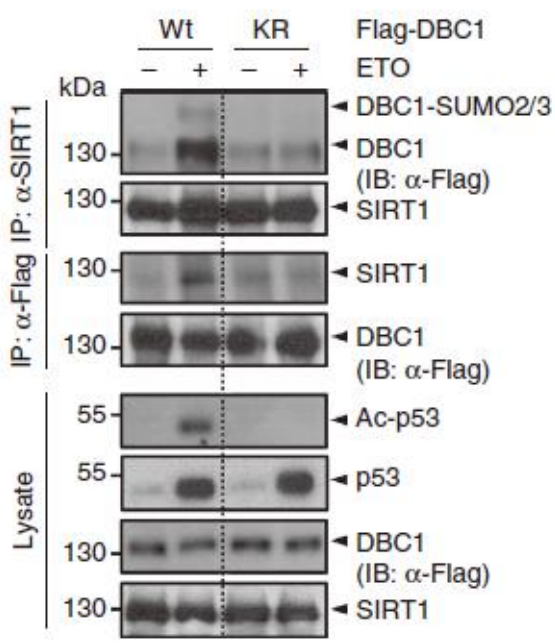

Wt
KR
Flag-DBC1
–
+
–
+
ETO
kDa
IP: α-SIRT1
IP: α-Flag
Lysate
130
130
130
130
55
55
130
130
DBC1-SUMO2/3
DBC1
(IB: α-Flag)
SIRT1
SIRT1
DBC1
(IB: α-Flag)
Ac-p53
p53
DBC1
(IB: α-Flag)
SIRT1

Figure 10. Etoposide-induced sumoylation of DBC1 increases its interaction with SIRT1.

HeLa cells expressing shNS or shSUMO2/3 were incubated for 36 h with and without Flag-SUMO3 in the presence and absence of etoposide. Their lysates were subjected to immunoprecipitation with anti-SIRT1 antibody followed by immunoblot analysis.'i' and 'e' denote shRNA insensitive and endogenous, respectively.

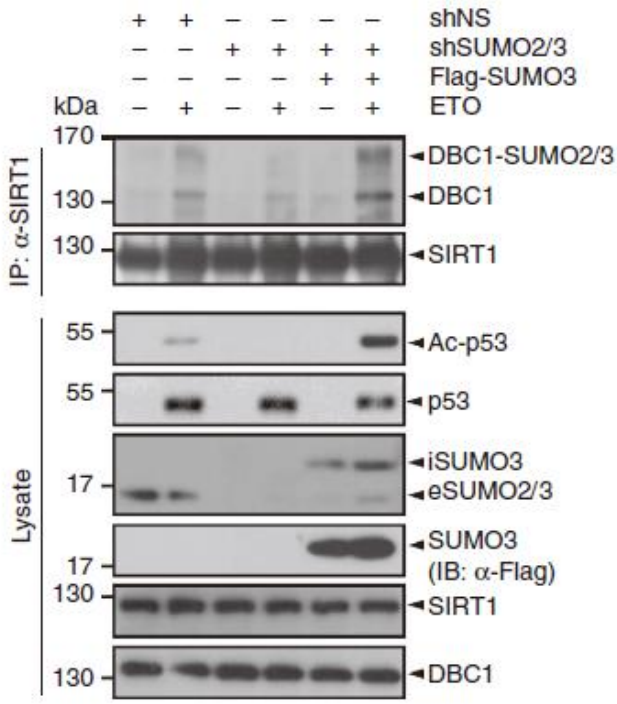
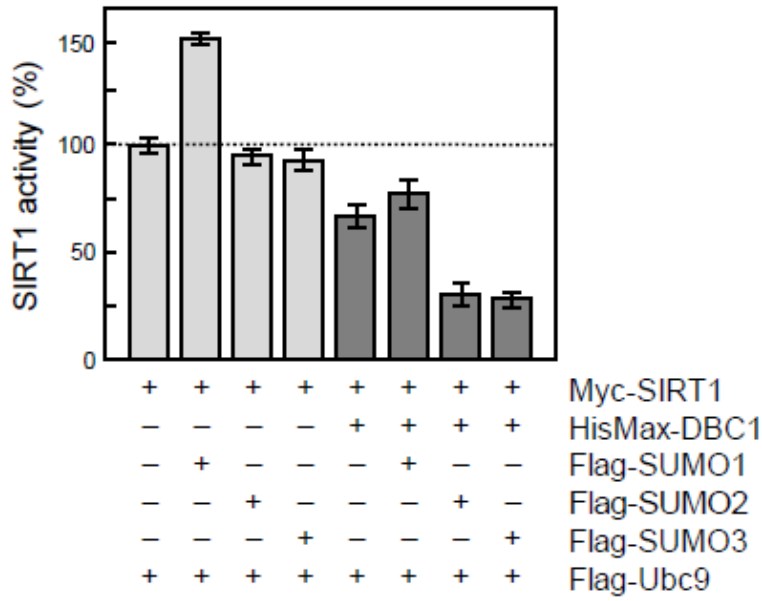

+ + − − − − shNS
− − + + + + shSUMO2/3
− − − − + + Flag-SUMO3
kDa − + − + − + ETO
170
130
130
IP: α-SIRT1
DBC1-SUMO2/3
DBC1
SIRT1
55
55
17
17
130
130
Lysate
Ac-p53
p53
iSUMO3
eSUMO2/3
SUMO3
(IB: α-Flag)
SIRT1
DBC1

Figure 11. Effects of DBC1 sumoylation on the SIRT1 activity and the NAD^+/NADH ratio.

(A) Myc-SIRT1 and Flag-Ubc9 were expressed in HEK293T cells with and without HisMax-DBC1 and/or each of SUMO isoforms. The deacetylase activity of SIRT1 was then assayed as described under Materials and Methods. The SIRT1 activity seen only with Myc-SIRT1 and Flag-Ubc9 was expressed as 100% and the others were as its relative values. Error bar, ± s.d. (n=3). (B) The cells were also subjected to determination of the NAD^+/NADH ratio. The NAD^+/NADH ratio seen only with Myc-SIRT1 and Flag-Ubc9 was expressed as 1.0 and the others were as its relative values. Error bar, ± s.d. (n=3).

A


SIRT1 activity (%)
150
100
50
0
+ + + + + + + + Myc-SIRT1
– – – – + + + + HisMax-DBC1
– + – – – + – – Flag-SUMO1
– – + – – – + – Flag-SUMO2
– – – + – – – + Flag-SUMO3
+ + + + + + + + Flag-Ubc9

B

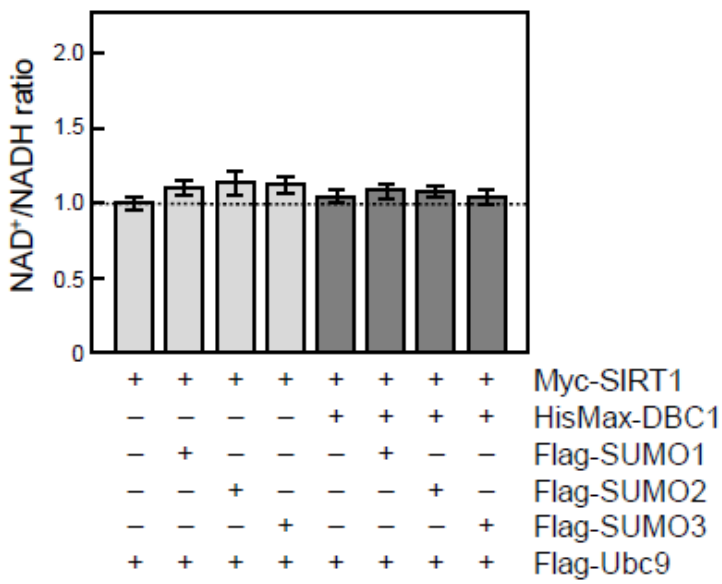
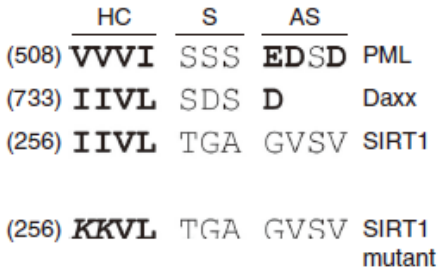
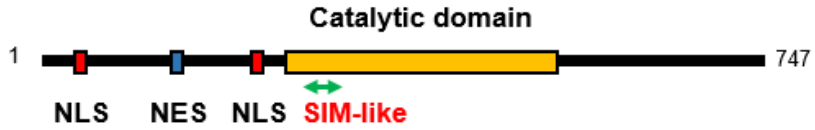

NAD+/NADH ratio
2.0
1.5
1.0
0.5
0
+ + + + + + + + Myc-SIRT1
– – – – + + + + HisMax-DBC1
– + – – – + – – Flag-SUMO1
– – + – – – + – Flag-SUMO2
– – – + – – – + Flag-SUMO3
+ + + + + + + + Flag-Ubc9

Figure 12. SIRT1 has a SIM-like sequence.

(A) The SUMO-interacting motifs (SIMs) of PML and Daxx were compared with a similar sequence in SIRT1. Two lle residues in the hydrophobic core (HC) sequence of SIRT1 were replaced by two Lys residues. ‘S’ and ‘AS’ indicate spacer and acidic stretch sequences, respectively. There are scheme of SIM-like sequence in SIRT1 protein. (B) Flag-tagged SUMO isoforms were expressed in HEK293T cells with Myc-tagged SIRT1 or its KKVL mutant. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot analysis.

A


HC
S
AS
(508) VVVI SSS EDSD PML
(733) IIVL SDS D Daxx
(256) IIVL TGA GVSV SIRT1
(256) KKVL TGA GVSV SIRT1 mutant


Catalytic domain
1
747
NLS
NES
NLS
SIM-like

B

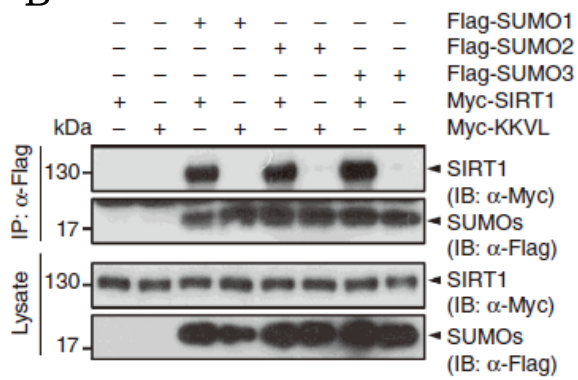

Flag-SUMO1
Flag-SUMO2
Flag-SUMO3
Myc-SIRT1
Myc-KKVL
kDa
IP: α-Flag
Lysate
130
17
SIRT1 (IB: α-Myc)
SUMOs (IB: α-Flag)
SIRT1 (IB: α-Myc)
SUMOs (IB: α-Flag)

Figure 13. Competition of SUMO3 with SUMO3-conjugated DBC1 for binding to SIRT1.

Purified Flag-SUMO3-conjugated HisMax-DBC1 and GST-SIRT1 proteins were incubated with increasing amounts of purified Flag-SUMO3 for 2 h at 4°C. The samples were then subjected to immunoprecipitation with anti-GST antibody followed by immunoblot analysis.

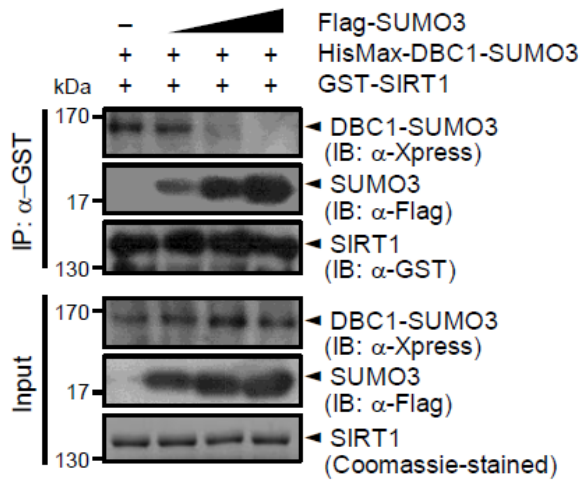
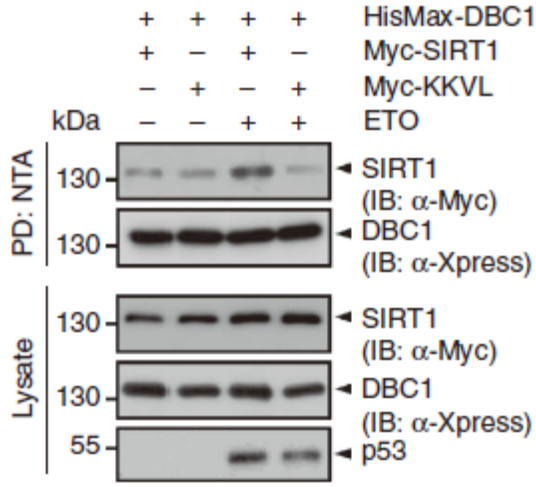

–
Flag-SUMO3
+ + + +
HisMax-DBC1-SUMO3
kDa
+ + + +
GST-SIRT1
170
17
130
IP: α–GST
DBC1-SUMO3
(IB: α-Xpress)
SUMO3
(IB: α-Flag)
SIRT1
(IB: α-GST)
170
17
130
Input
DBC1-SUMO3
(IB: α-Xpress)
SUMO3
(IB: α-Flag)
SIRT1
(Coomassie-stained)

Figure 14. SIM-like sequence of SIRT1 is essential for its binding to sumoylated DBC1.

(A) HisMax-DBC1 was expressed in HeLa cells with Myc-tagged SIRT1 or its KKVL mutant, and incubated with and without etoposide for 36 h. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot analysis. (B) HisMax-p53 and Flag-p300 were expressed in HeLa cells with Myc-tagged SIRT1 or its KKVL mutant. Cell lysates were subjected to immunoblot analysis.

A


+ + + + HisMax-DBC1
+ – + – Myc-SIRT1
– + – + Myc-KKVL
kDa – – + + ETO
PD: NTA
130
SIRT1
(IB: α-Myc)
130
DBC1
(IB: α-Xpress)
Lysate
130
SIRT1
(IB: α-Myc)
130
DBC1
(IB: α-Xpress)
55
p53

B

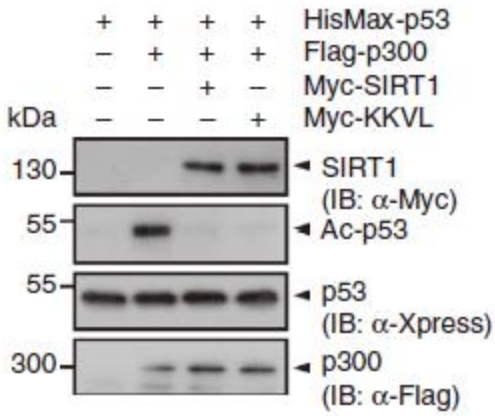

+ + + + HisMax-p53
– + + + Flag-p300
– – + – Myc-SIRT1
kDa – – – + Myc-KKVL
130
SIRT1
(IB: α-Myc)
55
Ac-p53
55
p53
(IB: α-Xpress)
300
p300
(IB: α-Flag)

Figure 15. DBC1, but not KR mutant, blocks the interaction of SIRT1 with p53.

HeLa cells expressing Flag-tagged DBC1 (Wt) or its K591R mutant (KR) were incubated with etoposide for 36 h. Their lysates were subjected to immunoprecipitation with pre-immune IgG or anti-SIRT1 antibody followed by immunoblot analysis.

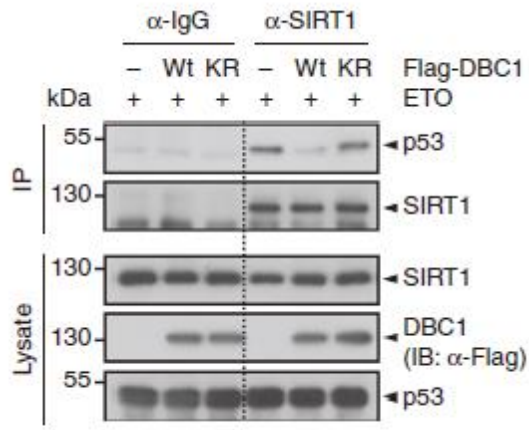

α-IgG
α-SIRT1
– Wt KR – Wt KR
Flag-DBC1
kDa + + + + + +
ETO
IP
55
p53
130
SIRT1
Lysate
130
SIRT1
130
DBC1
(IB: α-Flag)
55
p53

Figure 16. Knockdown of SUMO2/3 increases the interaction of SIRT1 with p53.

HeLa cells expressing shNS or shSUMO2/3 were incubated with etoposide for 36 h. Their lysates were subjected to immunoprecipitation with anti-p53 antibody followed by immunoblot analysis.

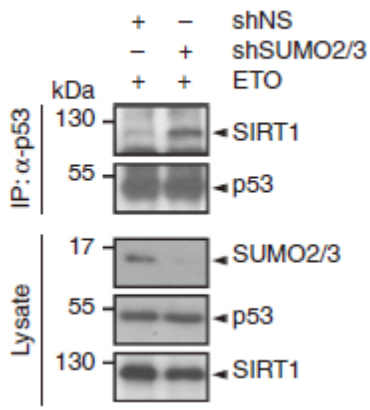

+ – shNS
– + shSUMO2/3
+ + ETO
kDa
IP: α-p53
130
SIRT1
55
p53
Lysate
17
SUMO2/3
55
p53
130
SIRT1

Figure 17. SUMO3 increases interaction of DBC1 with SIRT1, but not by KR mutant.

HisMax–DBC1 or its K591R mutant, Flag-Ubc9, HA-p53 and Myc-SIRT1 were expressed in HEK293T cells with increasing amounts of Flag-SUMO3. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody followed by immunoblot analysis.

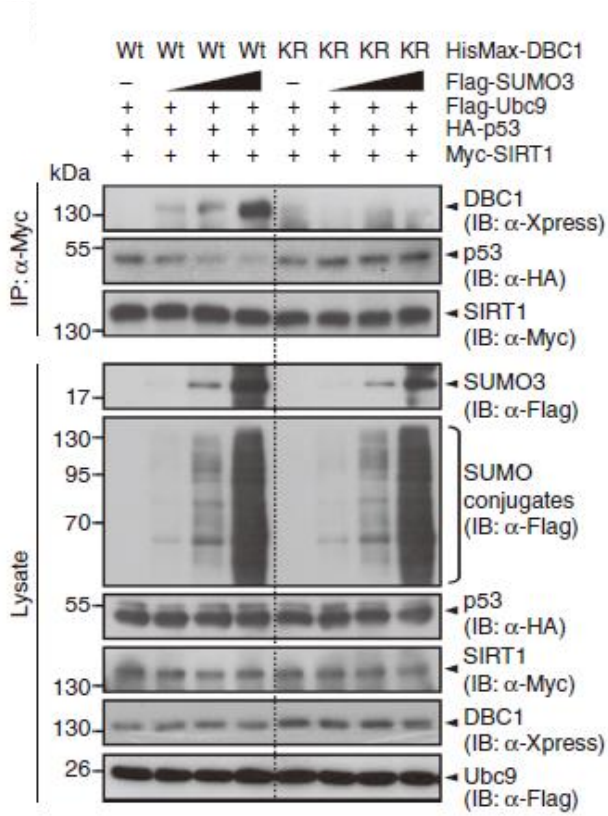

Wt Wt Wt Wt KR KR KR KR HisMax-DBC1
– Flag-SUMO3
+ + + + + + + + Flag-Ubc9
+ + + + + + + + HA-p53
+ + + + + + + + Myc-SIRT1
kDa
IP: α-Myc
130
55
130
DBC1 (IB: α-Xpress)
p53 (IB: α-HA)
SIRT1 (IB: α-Myc)
Lysate
17
130
95
70
55
130
130
26
SUMO3 (IB: α-Flag)
SUMO conjugates (IB: α-Flag)
p53 (IB: α-HA)
SIRT1 (IB: α-Myc)
DBC1 (IB: α-Xpress)
Ubc9 (IB: α-Flag)

Figure 18. PIAS3 specifically interacts with DBC1 among PIAS family.

HisMax-DBC1 was expressed in HEK293T cells with each of Flag-tagged PIAS1-4. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot analysis.

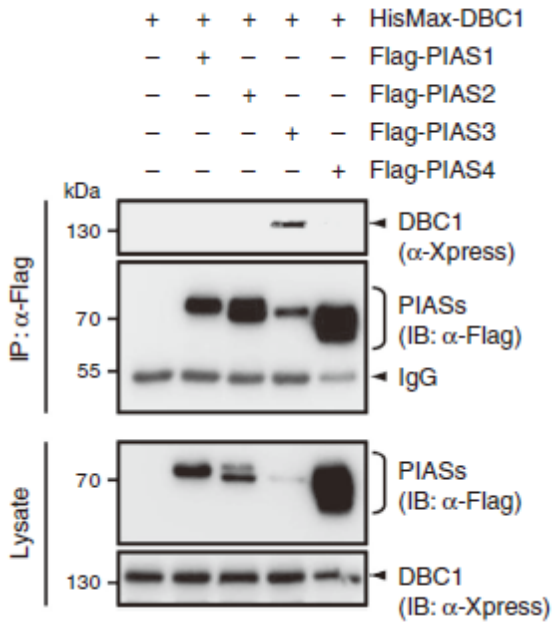

+ + + + + HisMax-DBC1
– + – – – Flag-PIAS1
– – + – – Flag-PIAS2
– – – + – Flag-PIAS3
– – – – + Flag-PIAS4
kDa
130
70
55
IP: α-Flag
DBC1
(α-Xpress)
PIASs
(IB: α-Flag)
IgG
70
130
Lysate
PIASs
(IB: α-Flag)
DBC1
(IB: α-Xpress)

Figure 19. Etoposide induces interaction of PIAS3 with DBC1.

HeLa cells incubated with and without etoposide for 36 h were subjected to immunoprecipitation with anti-DBC1 antibody followed by immunoblot analysis.

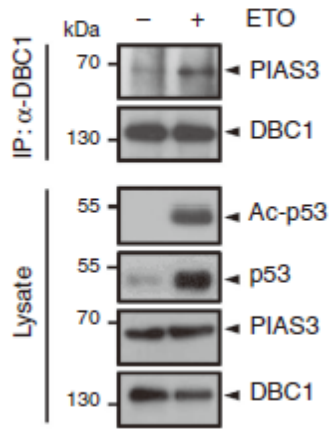

kDa
–
+
ETO
IP: α-DBC1
70
PIAS3
130
DBC1
Lysate
55
Ac-p53
55
p53
70
PIAS3
130
DBC1

Figure 20. Overexpression of PIAS3 increases DBC1 sumoylation.

Flag-tagged SUMO3 and Ubc9 were expressed in HEK293T cells with HisMax-DBC1 and/or Myc-PIAS3. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot analysis.

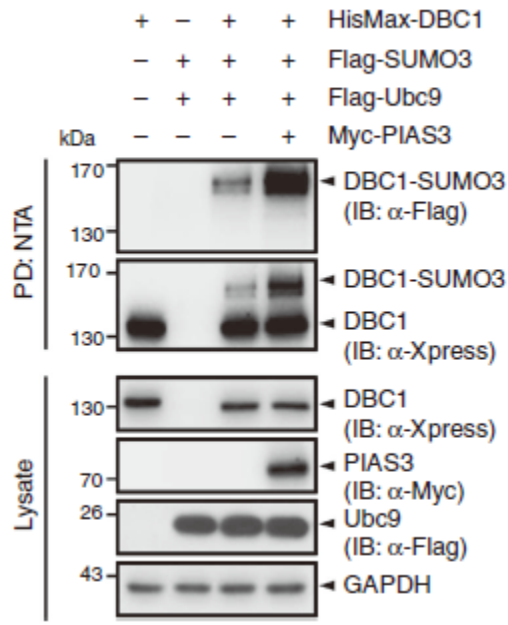

+ − + + HisMax-DBC1
− + + + Flag-SUMO3
− + + + Flag-Ubc9
kDa − − − + Myc-PIAS3
PD: NTA
170
130
DBC1-SUMO3
(IB: α-Flag)
170
130
DBC1-SUMO3
DBC1
(IB: α-Xpress)
Lysate
130
DBC1
(IB: α-Xpress)
70
PIAS3
(IB: α-Myc)
26
Ubc9
(IB: α-Flag)
43
GAPDH

Figure 21. Knockdown of PIAS3 prevents sumoylation of DBC1 with a reduction in etoposide-induced SIRT1-DBC1 interaction.

HeLa cells expressing shNS or shPIAS3 were incubated with and without etoposide for 36 h. Their lysates were subjected to immunoprecipitation with anti-DBC1 antibody followed by immunoblot analysis.

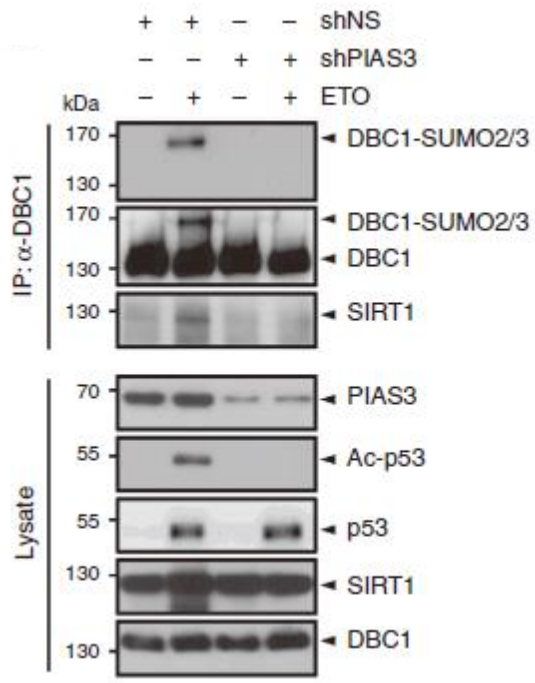

+ + − − shNS
− − + + shPIAS3
kDa − + − + ETO
IP: α-DBC1
170
130
DBC1-SUMO2/3
170
130
DBC1-SUMO2/3
DBC1
130
SIRT1
Lysate
70
PIAS3
55
Ac-p53
55
p53
130
SIRT1
130
DBC1

Figure 22. Knockdown of PIAS3 prevents etoposide-induced DBC1-SIRT1 interaction.

shNS and shPIAS3 were expressed in HeLa cells with and without Myc-PIAS3. After incubation with and without etoposide for 36 h, cell lysates were subjected to immunoprecipitation with anti-SIRT1 antibody followed by immunoblot analysis. 'i' and 'e' denote shRNA insensitive and endogenous, respectively. .

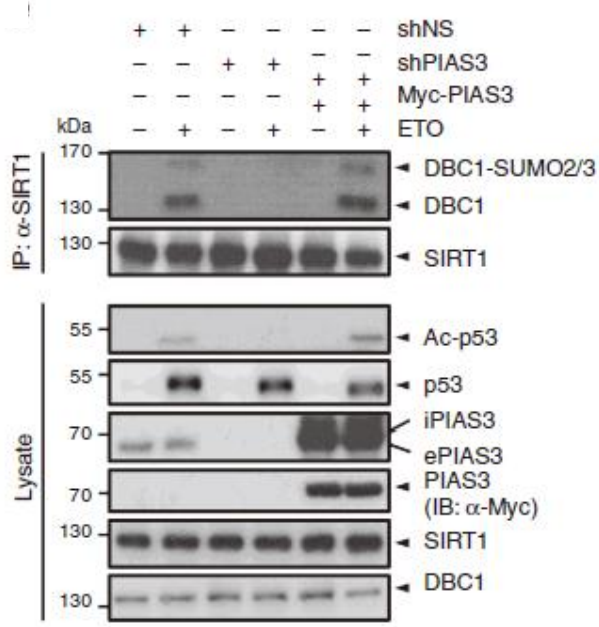
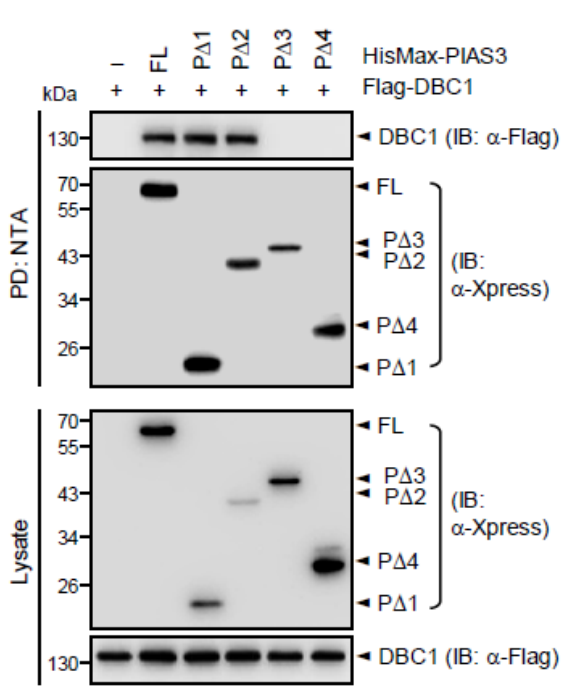

shNS
shPIAS3
Myc-PIAS3
ETO
kDa
IP: α-SIRT1
Lysate
170
130
130
55
55
70
70
130
130
DBC1-SUMO2/3
DBC1
SIRT1
Ac-p53
p53
iPIAS3
ePIAS3
PIAS3
(IB: α-Myc)
SIRT1
DBC1

Figure 23. Mapping the regions for the interaction between PIAS3 and DBC1.

(A) Deletions of PIAS3 were generated, tagged with HisMax to their N-termini, and expressed in HEK293T cells with Flag-DBC1. Cell lysates were subjected to pull-down with NTA resins followed by immunoblot analysis. (B) Deletions of DBC1 were generated, tagged with Flag to their N-termini, and expressed in HEK293T cells with Myc-PIAS3. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot analysis. 'FL' indicates full-length.

A


HisMax-PIAS3
Flag-DBC1
kDa
– + FL + PΔ1 + PΔ2 + PΔ3 + PΔ4
PD: NTA
130
70
55
43
34
26
DBC1 (IB: α-Flag)
FL
PΔ3
PΔ2
PΔ4
PΔ1
(IB: α-Xpress)
Lysate
130
DBC1 (IB: α-Flag)

B

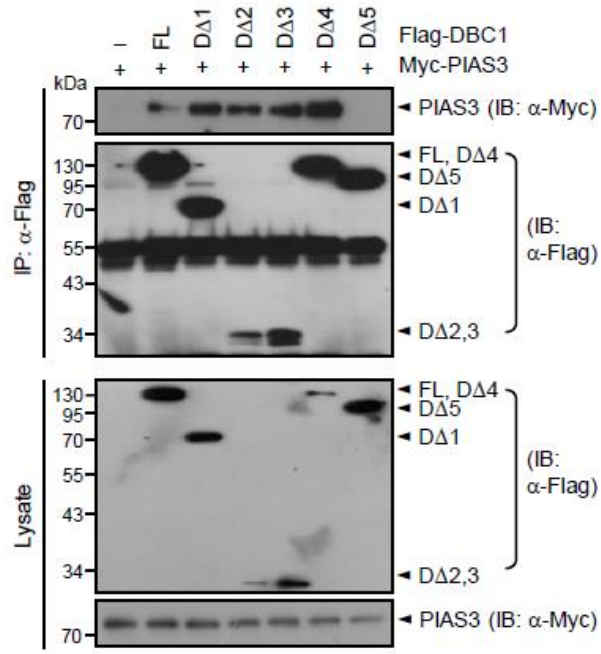

Flag-DBC1
Myc-PIAS3
kDa
– + FL + DΔ1 + DΔ2 + DΔ3 + DΔ4 + DΔ5
IP: α-Flag
70
PIAS3 (IB: α-Myc)
130
95
70
55
43
34
FL, DΔ4
DΔ5
DΔ1
DΔ2,3
(IB: α-Flag)
Lysate
PIAS3 (IB: α-Myc)

Figure 24. Interaction of SENPs with DBC1 under overexpression condition.

HisMax-DBC1 was expressed in HEK293T cells with each of SENP1, 2, 5 and 7 or SENP3 and 6. Cell lysates were subjected to immunoprecipitation with anti-Myc and anti-Flag antibodies followed by immunoblot analysis.

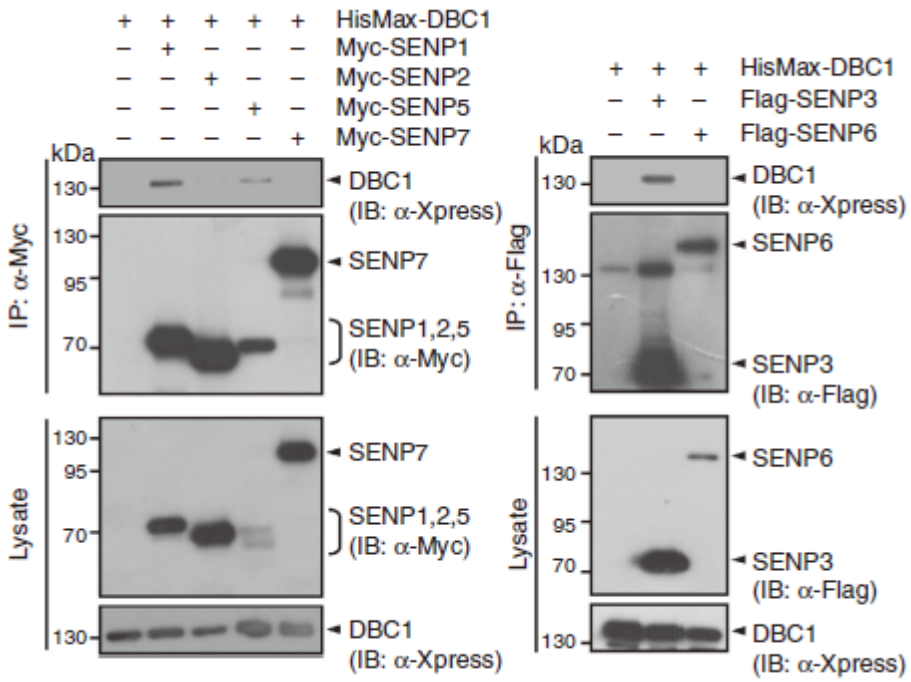

+ + + + + HisMax-DBC1
– + – – – Myc-SENP1
– – + – – Myc-SENP2
– – – + – Myc-SENP5
– – – – + Myc-SENP7
kDa
130
130
95
70
DBC1
(IB: α-Xpress)
SENP7
SENP1,2,5
(IB: α-Myc)
IP: α-Myc
Lysate
130
95
70
SENP7
SENP1,2,5
(IB: α-Myc)
130
DBC1
(IB: α-Xpress)
+ + + HisMax-DBC1
– + – Flag-SENP3
– – + Flag-SENP6
kDa
130
130
95
70
DBC1
(IB: α-Xpress)
SENP6
SENP3
(IB: α-Flag)
IP: α-Flag
Lysate
130
95
70
SENP6
SENP3
(IB: α-Flag)
130
DBC1
(IB: α-Xpress)

Figure 25. Etoposide decreases interaction of SENP1 and DBC1.

HeLa cells incubated with and without etoposide for 36 h were subjected to immunoprecipitation with andi-DBC1 antibody followed by immunoblot analysis.

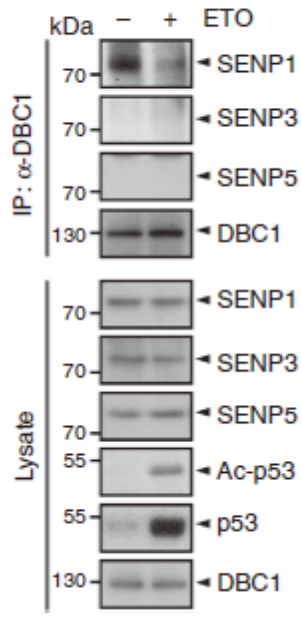

kDa
–
+
ETO
IP: α-DBC1
70
SENP1
70
SENP3
70
SENP5
130
DBC1
Lysate
70
SENP1
70
SENP3
70
SENP5
55
Ac-p53
55
p53
130
DBC1

Figure 26. Overexpression of SENP1, but not its C603S mutant, leads to complete desumoylation of DBC1.

HisMax-DBC1, Flag-Ubc9 and Flag-SUMO3 were expressed in HEK293T cells with Myc-tagged SENP1 or its C603S mutant. Cell lysates were subjected to pull-down with NTA-resins followed by immunoblot analysis.

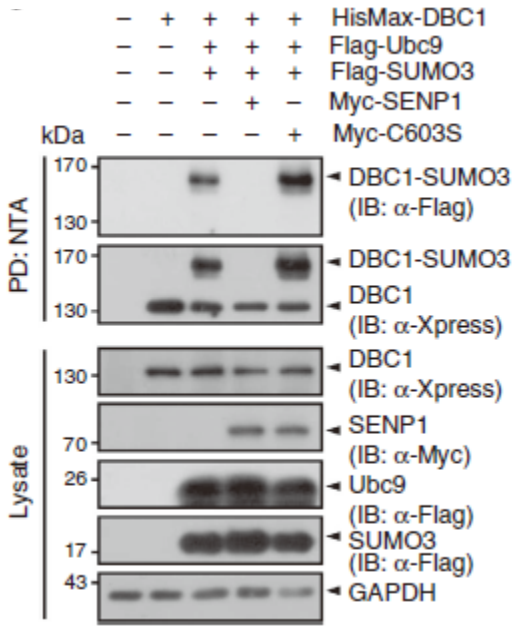

HisMax-DBC1
Flag-Ubc9
Flag-SUMO3
Myc-SENP1
Myc-C603S
kDa
PD: NTA
170
130
DBC1-SUMO3
(IB: α-Flag)
DBC1-SUMO3
DBC1
(IB: α-Xpress)
Lysate
DBC1
(IB: α-Xpress)
SENP1
(IB: α-Myc)
Ubc9
(IB: α-Flag)
SUMO3
(IB: α-Flag)
GAPDH
70
26
17
43

Figure 27. Knockdown of SENP1 increases etoposide-induced DBC1 sumoylation and interaction between DBC1 and SIRT1.

HeLa cells expressing shNS or shSENP1 were incubated with and without etoposide for 36 h. Their lysates were subjected to immunoprecipitation with anti-DBC1 antibody followed by immunoblot analysis.

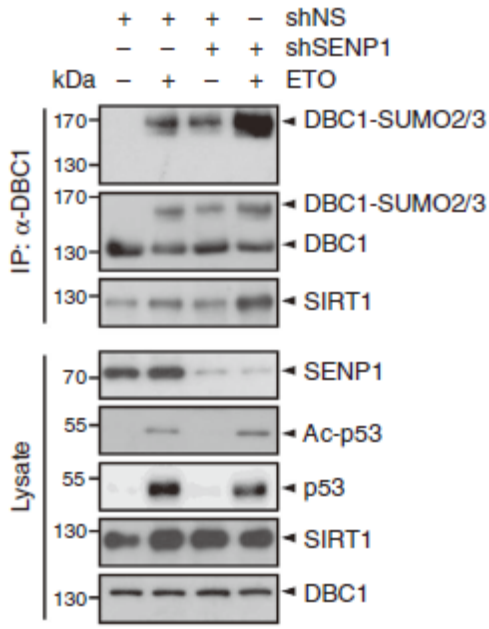

+ + + − shNS
− − + + shSENP1
kDa − + − + ETO
IP: α-DBC1
170
130
DBC1-SUMO2/3
170
130
DBC1-SUMO2/3
DBC1
130
SIRT1
Lysate
70
SENP1
55
Ac-p53
55
p53
130
SIRT1
130
DBC1

Figure 28. Complementation of shRNA-insensitive SENP1 reverses the effects of SENP1 depletion on the DBC1-SIRT1 interaction.

shNS and shSENP1 were expressed in HeLa cells with and without Myc-SENP1. They were incubated with and without etoposide for 36 h. Cell lysates were subjected to immunoprecipitation with anti-SIRT1 antibody followed by immunoblot analysis. 'i' and 'e' denote shRNA insensitive and endogenous, respectively.

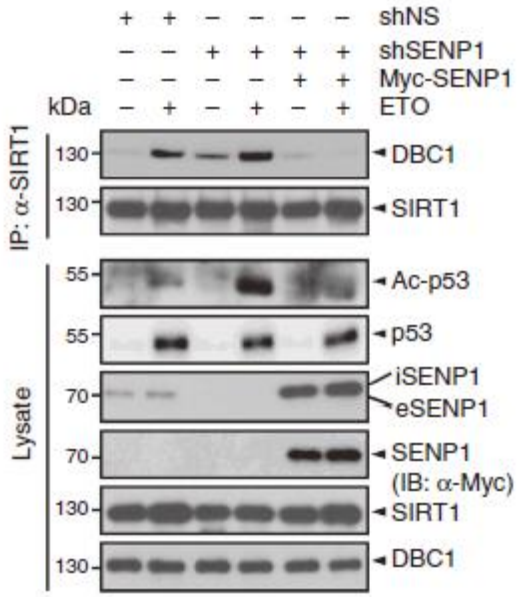
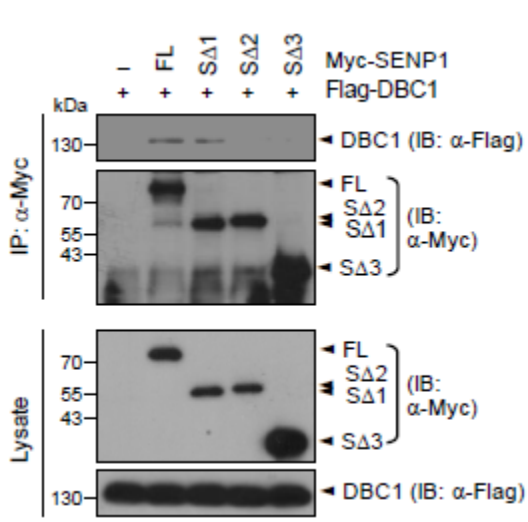

shNS
shSENP1
Myc-SENP1
ETO
kDa
IP: α-SIRT1
130
DBC1
130
SIRT1
Lysate
55
Ac-p53
55
p53
70
iSENP1
eSENP1
70
SENP1
(IB: α-Myc)
130
SIRT1
130
DBC1

Figure 29. Mapping the regions for the interaction between SENP1 and DBC1.

(A) Deletions of SENP1 were generated, tagged with Myc to their N-termini, and expressed in HEK293T cells with Flag-DBC1. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody followed by immunoblot analysis. (B) Deletions of DBC1 were generated, tagged with Flag to their N-termini, and expressed in HEK293T cells with Myc-SENP1. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot analysis. 'FL' indicates full-length.

A


Myc-SENP1
Flag-DBC1
kDa
IP: α-Myc
Lysate
DBC1 (IB: α-Flag)
FL
SΔ2
SΔ1
SΔ3
(IB: α-Myc)

B

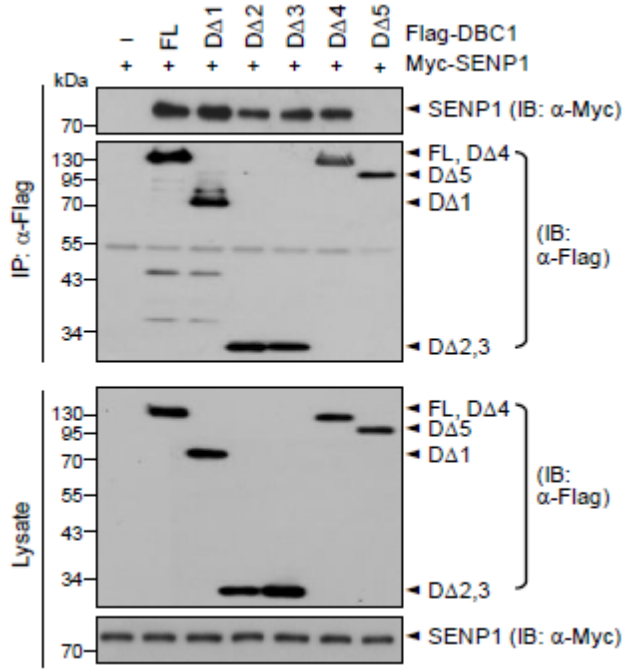
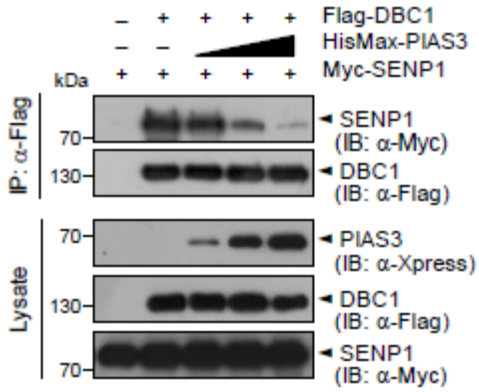

Flag-DBC1
Myc-SENP1
kDa
IP: α-Flag
Lysate
SENP1 (IB: α-Myc)
FL, DΔ4
DΔ5
DΔ1
DΔ2,3
(IB: α-Flag)

Figure 30. PIAS3 competes with SENP1 for binding to DBC1.

Flag-DBC1 and Myc-SENP1 were expressed in HEK293T cells with increasing amounts of HisMax-PIAS3 (top). HisMax-DBC1 and Flag-PIAS3 were expressed with increasing amounts of Myc-SENP1 (bottom). Cell lysates were subjected to immunoprecipitation with anti-Flag antibody (top) and pull-down with NTA resins (bottom) followed by immunoblot analysis.


Flag-DBC1
HisMax-PIAS3
Myc-SENP1
kDa
IP: α-Flag
70
130
SENP1
(IB: α-Myc)
DBC1
(IB: α-Flag)
Lysate
70
130
70
PIAS3
(IB: α-Xpress)
DBC1
(IB: α-Flag)
SENP1
(IB: α-Myc)

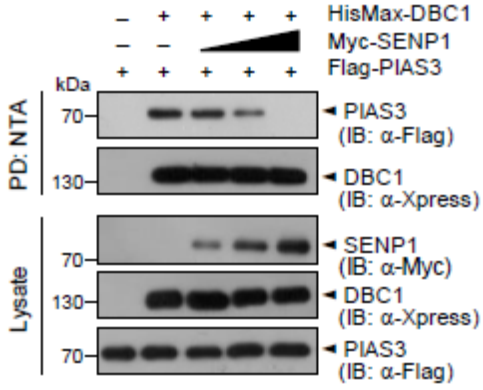

HisMax-DBC1
Myc-SENP1
Flag-PIAS3
kDa
PD: NTA
70
130
PIAS3
(IB: α-Flag)
DBC1
(IB: α-Xpress)
Lysate
70
130
70
SENP1
(IB: α-Myc)
DBC1
(IB: α-Xpress)
PIAS3
(IB: α-Flag)

Figure 31. DBC1 phosphorylation influences its sumoylation.

Flag-tagged SUMO3 and Ubc9 were expressed in HEK293T cells with HisMax-tagged DBC1, T454A or T454D. Cell lysates subjected to pull-down with NTA-resins followed by immunoblot analysis..

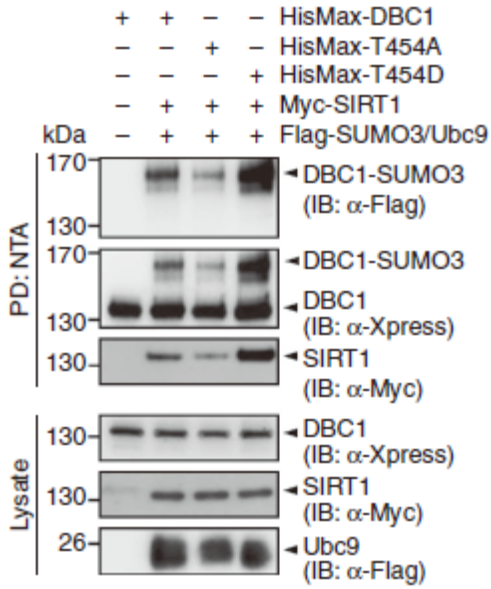

+ + − − HisMax-DBC1
− − + − HisMax-T454A
− − − + HisMax-T454D
− + + + Myc-SIRT1
kDa − + + + Flag-SUMO3/Ubc9
PD: NTA
170
130
DBC1-SUMO3
(IB: α-Flag)
170
130
DBC1-SUMO3
DBC1
(IB: α-Xpress)
130
SIRT1
(IB: α-Myc)
Lysate
130
DBC1
(IB: α-Xpress)
130
SIRT1
(IB: α-Myc)
26
Ubc9
(IB: α-Flag)

Figure 32. DBC1 phosphorylation switches its binding partner from SENP1 to PIAS3.

shRNA-insensitive Flag-tagged DBC1, T454A or T454D was expressed in HeLa cells that had been stably transfected with shNS or shDBC1. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot analysis.

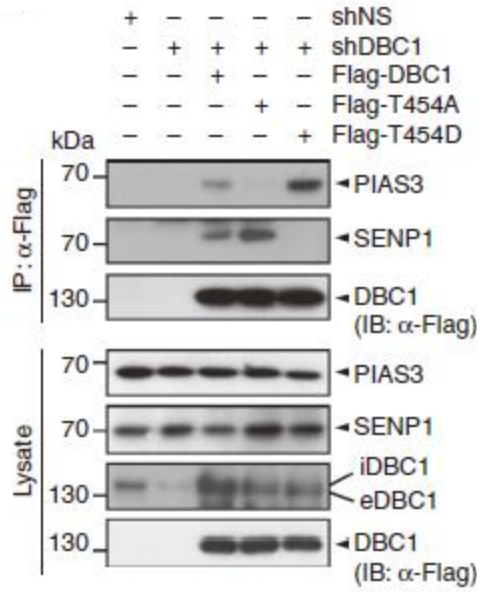

+ − − − − shNS
− + + + + shDBC1
− − + − − Flag-DBC1
− − − + − Flag-T454A
kDa − − − − + Flag-T454D
IP: α-Flag
70
PIAS3
70
SENP1
130
DBC1
(IB: α-Flag)
Lysate
70
PIAS3
70
SENP1
iDBC1
130
eDBC1
130
DBC1
(IB: α-Flag)

Figure 33. Caffeine blocks etoposide-induced DBC1 sumoylation and its interaction with SIRT1.

HeLa cells were incubated with etoposide and caffeine for 36 h. Cell lysates were subjected to immunoprecipiation with anti-DBC1 antibody followed by immunoblot analysis.

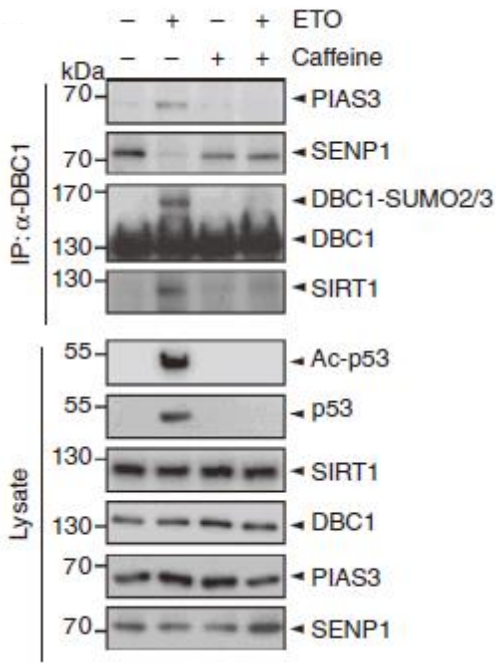
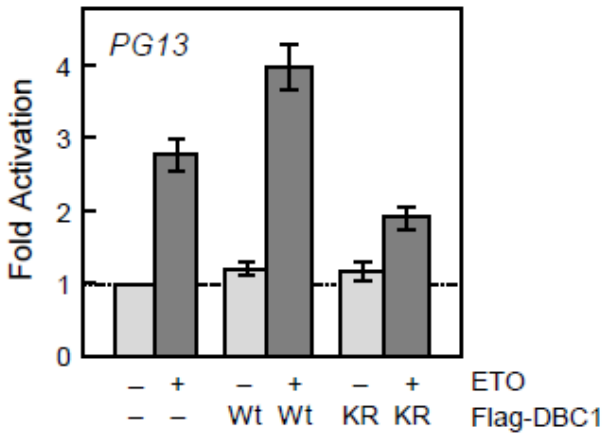

– + – + ETO
– – + + Caffeine
kDa
70
PIAS3
70
SENP1
170
DBC1-SUMO2/3
130
DBC1
130
SIRT1
IP: α-DBC1
55
Ac-p53
55
p53
130
SIRT1
130
DBC1
70
PIAS3
70
SENP1
Lysate

Figure 34. Overexpression of DBC1, but not KR mutant, increases p53 transactivity.

Flag-DBC1 (Wt) or its K591R mutant (KR) was expressed in HeLa cells with *PG13-LUC* (top) and *BAX-LUC* (bottom). After incubation with and without etoposide for 36 h, cells were subjected to assay for the luciferase activity. The activity seen without any treatment was expressed as 1.0 and the others were expressed as its relative values. Error bar, ± s.d. (n=3).


PG13
Fold Activation
0
1
2
3
4
– + – + – + ETO
– – Wt Wt KR KR Flag-DBC1

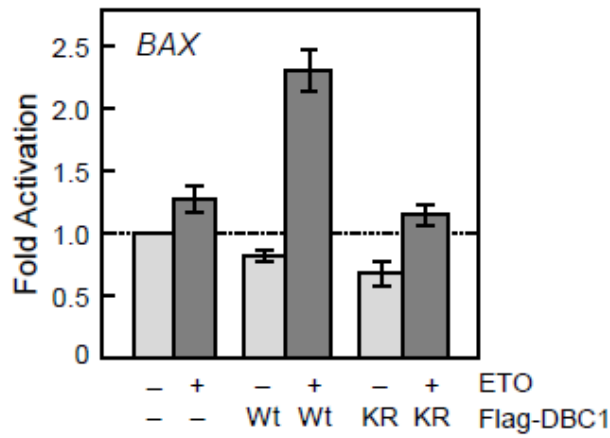
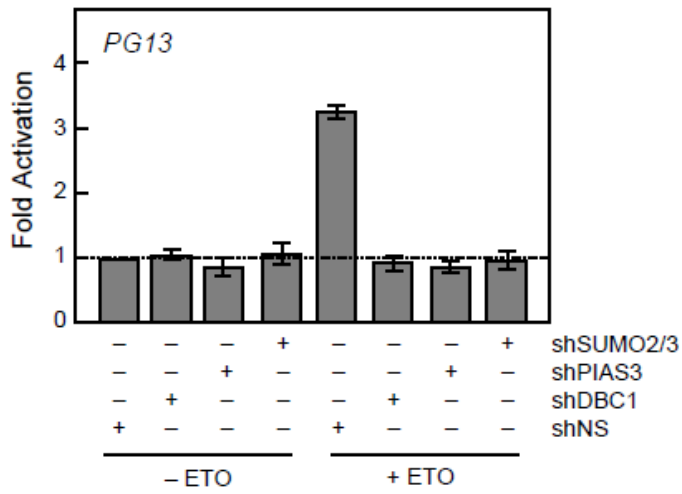

BAX
Fold Activation
0
0.5
1.0
1.5
2.0
2.5
– + – + – + ETO
– – Wt Wt KR KR Flag-DBC1

Figure 35. Knockdown of PIAS3 or SUMO2/3 blocks p53 transactivation.

shNS, shDBC1, shPIAS3 or shSUMO2/3 was expressed in HeLa cells with PG13-Luc (top) or BAX-Luc (bottom). After incubation with and without etoposide for 36 h, cells were subjected to assay for the luciferase activity. The activity seen without any treatment was expressed as 1.0 and the others were expressed as its relative values. Error bar, ± s.d. (n=3).


PG13
Fold Activation
0
1
2
3
4
– – – + – – – + shSUMO2/3
– – + – – – + – shPIAS3
– + – – – + – – shDBC1
+ – – – + – – – shNS
– ETO
+ ETO

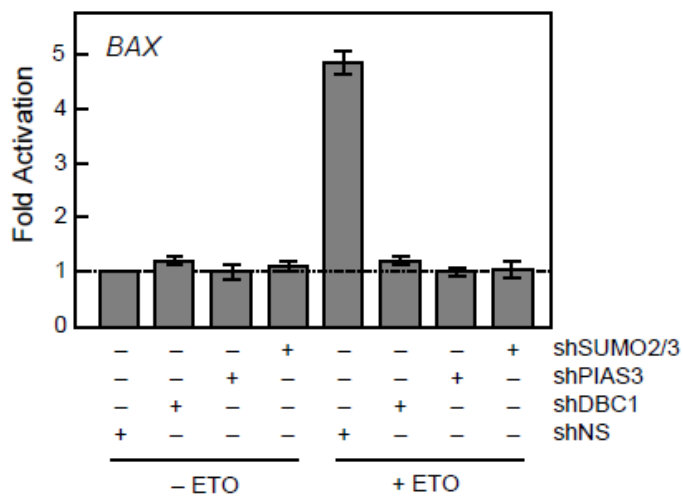
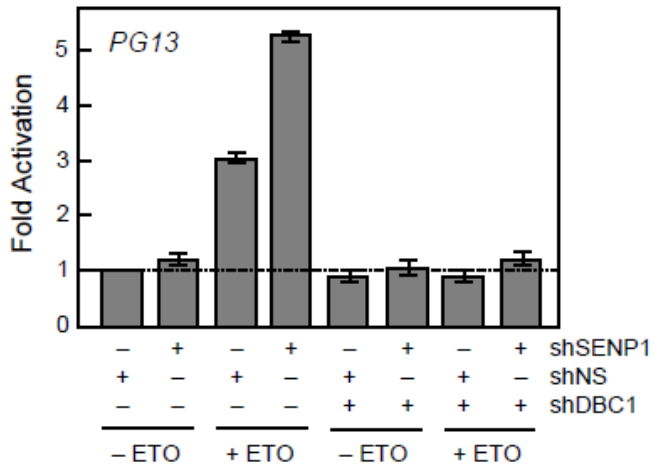

BAX
Fold Activation
0
1
2
3
4
5
– – – + – – – + shSUMO2/3
– – + – – – + – shPIAS3
– + – – – + – – shDBC1
+ – – – + – – – shNS
– ETO
+ ETO

Figure 36. Knockdown of SENP1 increases p53 transactivation.

shNS or shDBC1 and/or shSENP1 was expressed in HeLa cells with PG13-Luc (top) or BAX-Luc (bottom). After incubation with and without etoposide for 36 h, cells were subjected to assay for the luciferase activity. The activity seen without any treatment was expressed as 1.0 and the others were expressed as its relative values. Error bar, ± s.d. (n=3).


PG13
Fold Activation
0
1
2
3
4
5
– + – + – + – + shSENP1
+ – + – + – + – shNS
– – – – + + + + shDBC1
– ETO
+ ETO
– ETO
+ ETO

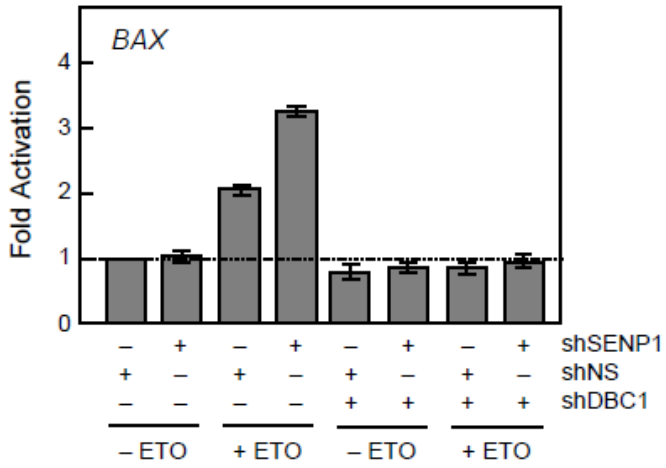

BAX
Fold Activation
0
1
2
3
4
– + – + – + – + shSENP1
+ – + – + – + – shNS
– – – – + + + + shDBC1
– ETO
+ ETO
– ETO
+ ETO

Figure 37. Overexpression of DBC1, but not by KR mutant, increases p53-mediated apoptotic genes.

shRNA-insensitive Flag-tagged DBC1 or its K591R mutant was expressed in HeLa cells that had been stably transfected with shNS or shDBC1. After incubation with and without etoposide for 36 h, they were subjected to immunoblot with anti-DBC1, anti-acetyl p53, anti-cleaved PARP1 and anti-cleaved caspase-9 antibodies.

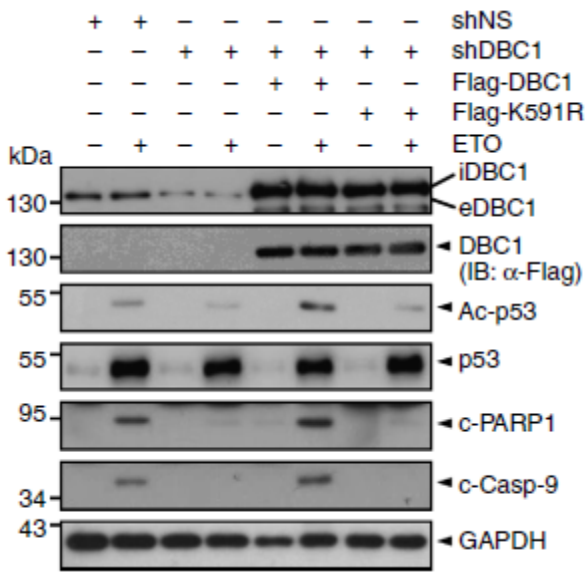

+ + − − − − − − shNS
− − + + + + + + shDBC1
− − − − + + − − Flag-DBC1
− − − − − − + + Flag-K591R
− + − + − + − + ETO
kDa
130
iDBC1
eDBC1
130
DBC1
(IB: α-Flag)
55
Ac-p53
55
p53
95
c-PARP1
34
c-Casp-9
43
GAPDH

Figure 38. Knockdown of SENP1 increases p53-mediated apoptotic genes.

shNS or shDBC1 was expressed in HeLa cells with or without shSENP1. Cells were then incubated in the presence and absence of etoposide for 36 h. Cell lysates were subjected to immunoblot with anti-DBC1, anti-SENP1, anti-acetyl p53, anti-cleaved PARP1 and anti-cleaved caspase-9 antibodies.

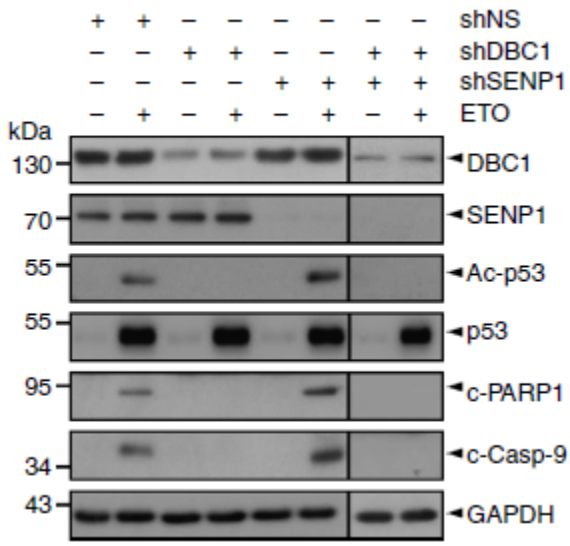

+ + − − − − − − shNS
− − + + − − + + shDBC1
− − − − + + + + shSENP1
− + − + − + − + ETO
kDa
130
70
55
55
95
34
43
DBC1
SENP1
Ac-p53
p53
c-PARP1
c-Casp-9
GAPDH

Figure 39. Knockdown of SUMO2/3 or PIAS3 decreases p53-mediated apoptotic genes.

shSUMO2/3 or shPIAS3 was expressed in HeLa cells with Flag-SUMO3 or Myc-PIAS3. After incubation with and without etoposide for 36 h, cell lysates were subjected to immunoblot with anti-DBC1, anti-SUMO2/3, anti-PIAS3, anti-acetyl p53, anti-cleaved PARP1 and anti-cleaved caspase-9 antibodies.

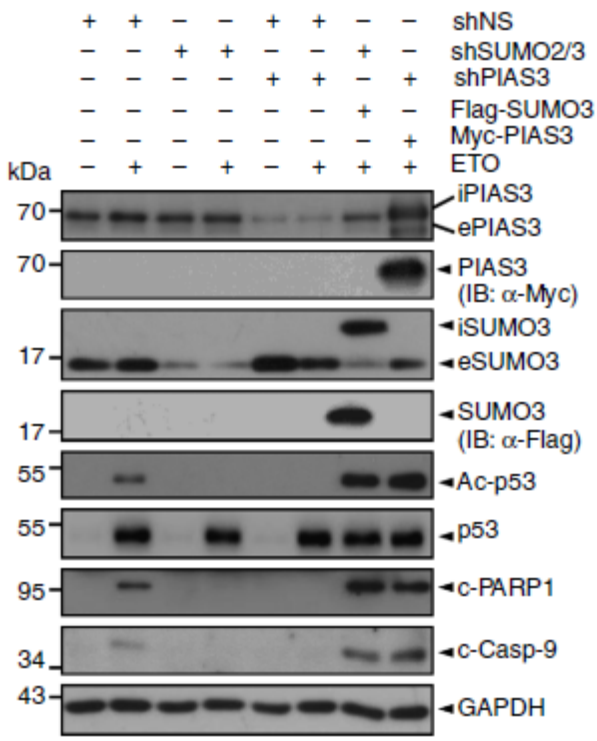

+ + − − + + − − shNS
− − + + − − + − shSUMO2/3
− − − − + + − + shPIAS3
− − − − − − + − Flag-SUMO3
− − − − − − − + Myc-PIAS3
kDa − + − + − + + + ETO
70
iPIAS3
ePIAS3
70
PIAS3
(IB: α-Myc)
17
iSUMO3
eSUMO3
17
SUMO3
(IB: α-Flag)
55
Ac-p53
55
p53
95
c-PARP1
34
c-Casp-9
43
GAPDH

Figure 40. Overexpression of DBC1, but not by KR mutant, increases the number of TUNEL-positive cells.

HeLa cells expressing Flag-DBC1 or its K591R mutant were incubated with and without etoposide for 36 h. They were then subjected to TUNEL assay. Scale bars, 20 μm.

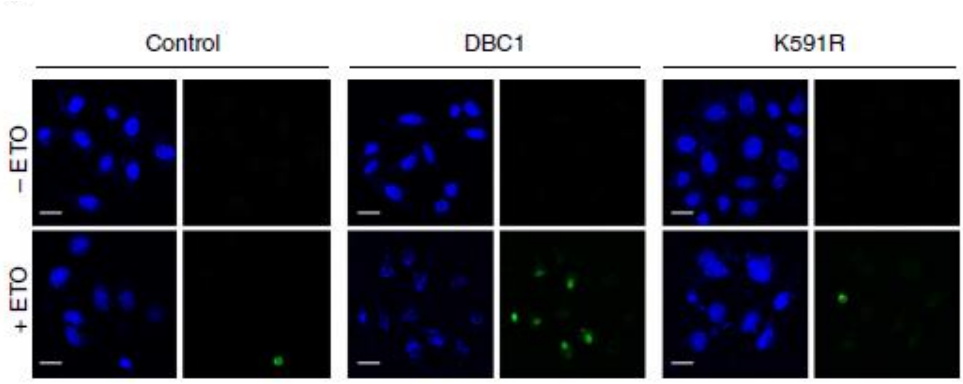

Control
DBC1
K591R
– ETO
+ ETO

Figure 41. Knockdown of SUMO2/3 or PIAS3 decreases the number of TUNEL-positive cells, but Knockdown of SENP1 increases it.

shNS or shDBC1 was expressed HeLa cells with shNS, shSUMO2/3, shPIAS3, shSENP1. After incubation with and without etoposide, cells were then subjected to TUNEL assay. Scale bars, 20 μm.

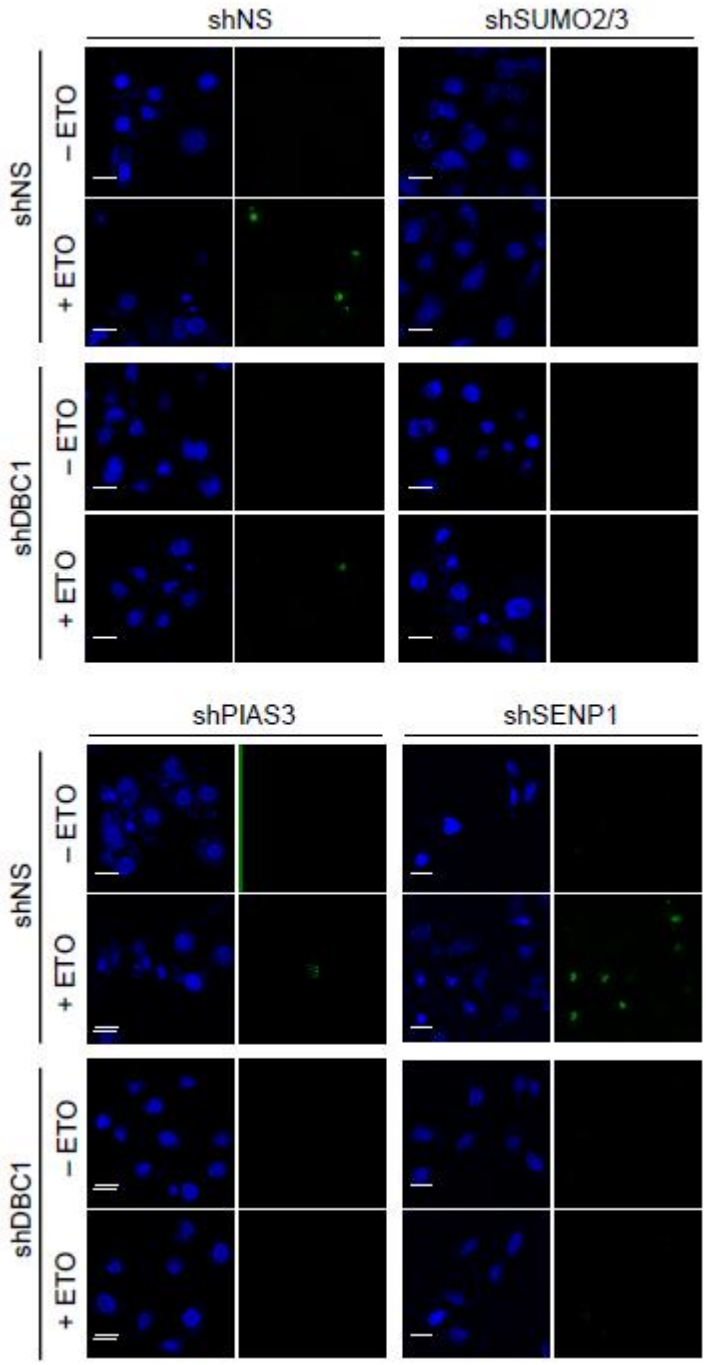
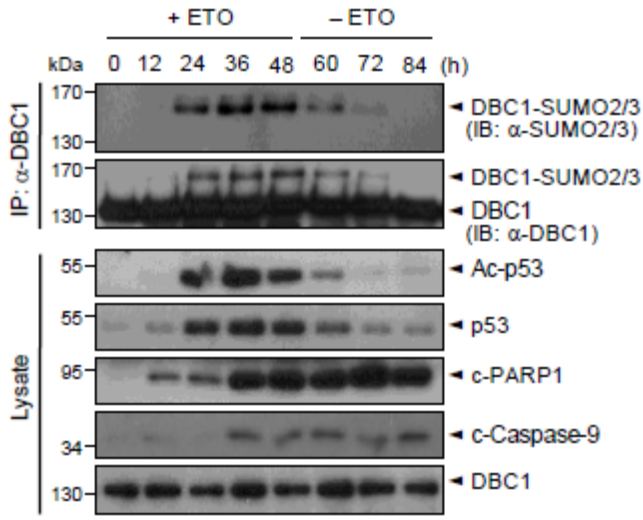

shNS
shSUMO2/3
shNS
– ETO
+ ETO
shDBC1
– ETO
+ ETO
shPIAS3
shSENP1
shNS
– ETO
+ ETO
shDBC1
– ETO
+ ETO

Figure 42. Effects of etoposide withdrawal on DBC1 sumoylation and apoptosis.

Etoposide was treated to HeLa cells for increasing periods up to 48 h. After the incubation, cells were further cultured for the next 36 h in fresh media in the absence of the drug. Cell lysates obtained at each time point were subjected to immunoprecipitation anti-DBC1 antibody followed by immunoblot analysis.

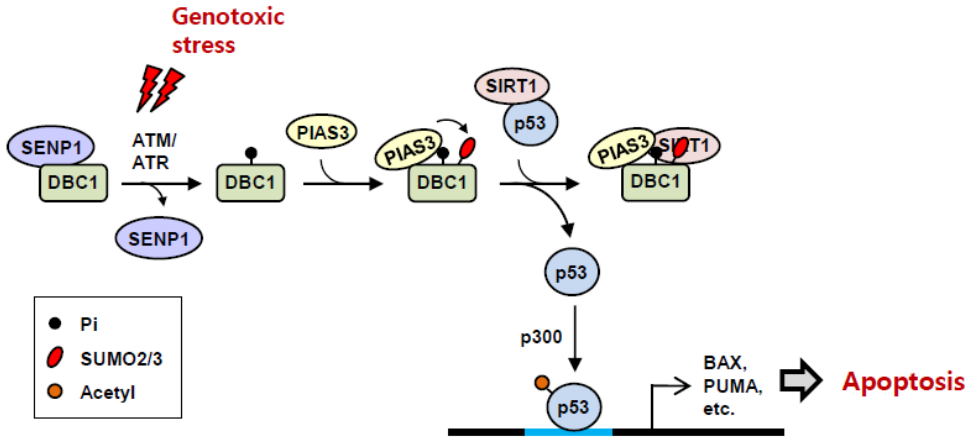

+ ETO
– ETO
kDa
0 12 24 36 48 60 72 84 (h)
IP: α-DBC1
170
130
DBC1-SUMO2/3
(IB: α-SUMO2/3)
170
130
DBC1-SUMO2/3
DBC1
(IB: α-DBC1)
Lysate
55
Ac-p53
55
p53
95
c-PARP1
34
c-Caspase-9
130
DBC1

DISCUSSION

Based on the present findings, I propose a model for the role of DBC1 sumoylation in the control of p53-mediated apoptosis in response to DNA damage (Figure 43). DBC1 is normally bound to SENP1. Under genotoxic stress, however, DBC1 becomes phosphorylated at Thr454 by the ATM/ATR kinases (Yuan et al, 2012; Zannini et al, 2012) and this phosphorylation switches the binding partner of DBC1 from SENP1 to PIAS3, leading to its sumoylation. DBC1 sumoylation markedly increases the DBC1-SIRT1 interaction and this tight binding displaces p53 from SIRT1, allowing acetylation (e.g., by p300) and transactivation of p53 for the expression of its downstream genes, such as BAX and PUMA, which induce apoptotic cell death. Interestingly, DBC1 was modified by SUMO2/3, but not by SUMO1, despite the fact that PIAS3 is capable of modifying other target proteins by SUMO1, such as hnRNP-K (Lee et al, 2012). Moreover, SIRT1 has a SIM-like sequence, which can bind SUMO2/3, suggesting

Figure 43. A model for the role of DBC1 sumoylation in p53-mediated apoptosis in response to DNA damage.

DBC1 is normally bound to SENP1. Under genotoxic stress, DBC1 is phosphorylated at Thr454 by the ATM/ATR kinases and this phosphorylation switches the binding partner of DBC1 from SENP1 to PIAS3, leading to its sumoylation. DBC1 sumoylation increases the DBC1-SIRT1 interaction and this tight binding displaces p53 from SIRT1, allowing acetylation and transactivation of p53 for the expression of its downstream genes, such as BAX, which induces apoptotic cell death.


Genotoxic stress
SENP1
DBC1
ATM/ ATR
SENP1
DBC1
PIAS3
PIAS3
DBC1
SIRT1
p53
PIAS3
SIRT1
DBC1
p53
p300
p53
BAX, PUMA, etc.
Apoptosis
Pi
SUMO2/3
Acetyl

that SUMO2/3 conjugated to DBC1 increases the affinity of the inhibitor protein to SIRT1. Collectively, post-translational modifications of DBC1 by the sequential actions of ATM/ATR kinases and PIAS3 play a crucial role in the control of the DBC1-SIRT1 interaction for p53-mediated apoptosis under genotoxic stress.

SUMO2/3, unlike SUMO1, is known to form poly-SUMO chains. However, DBC1 was found to be modified by a single molecule of SUMO2/3 (mono-SUMO2/3-ylated), but not by its polymeric chain. Likewise, Drp1 and ROR alpha have been shown to be mono-SUMO2/3-ylated (Guo et al, 2013; Hwang et al, 2009). Thus, it seems likely that SUMO2/3 can be conjugated to target proteins not only as polymeric chain(s) but also as a single molecule, although it remains unknown how the chain length is regulated.

Of interest was the finding that SENP1 and PIAS3 bind to the same N-terminal region of DBC1 and this competitive binding can be switched by ATM/ATR-mediated phosphorylation of DBC1. However, it remains unknown how the phosphorylation of DBC1 at Thr454, which is located distal to the binding region (the amino acids 1-243) of SENP1 and PIAS3, could influence the interaction of DBC1 with the SUMO-

modifying enzymes. Possibly in the 3D structure of DBC1, Thr454 may be in close proximity to the binding site for SENP1 and PIAS3, and when the threonine residue is phosphorylated, the negative charge might provide a better structural atmosphere for the interaction of DBC1 with PIAS3, but not with SENP1. Recently, it has been reported that ATM-mediated phosphorylation of DBC1 at Thr454 provides a second binding site for SIRT1, leading to inhibition of the deacetylase activity (Yuan et al, 2012). Notably, DBC1 is capable of forming a ternary complex with PIAS3 and SIRT1 upon treatment with etoposide and this complex formation could be prevented by co-treatment with caffeine, an inhibitor of the ATM/ATR kinases (Figure 33). Thus, it appears possible that DBC1 phosphorylation plays a dual role: one in providing a second binding site for SIRT1 and the other in promotion of PIAS3 binding to DBC1 for sumoylation, which further increases the DBC1-SIRT1 interaction via the SIM-like sequence of the deacetylase.

Significantly, it has been reported that SIRT1 can be modified by SUMO1 and this modification increases its deacetylase activity, leading to p53 inactivation and cell

survival (Yang et al, 2007). Under stress conditions, however, SENP1 inactivates SIRT1 by desumoylation and in turn activates p53 for stress-induced apoptosis. Therefore, SIRT1 sumoylation was suggested to act as a molecular switch that tips the balance from survival to death when DNA is damaged (Yang et al, 2007), although it was not determined whether SIRT1 sumoylation influences its ability to interact with DBC1. Interestingly, the same SENP1 enzyme is utilized for desumoylation of both SIRT1 and DBC1, although the enzyme removes SUMO1 from SIRT1 and SUMO2/3 from DBC1. However, SENP1-mediated desumoylation of SIRT1 and DBC1 appears to oppositely impact on cell fate. Whereas the action of SENP1 on SIRT1 promotes stress-induced apoptosis by inactivating the deacetylase activity, its action on DBC1 allows cells to survive by inhibiting the DBC1-SIRT1 interaction under unstressed conditions. DBC1 may sequester SENP1 from SIRT1 under normal conditions (Figure 25) and thereby SIRT1 would maintain its sumoylated state for p53 deacetylation. However, when DBC1 is phosphorylated in response to DNA damage, SENP1 bound to DBC1 would be replaced by PIAS3, and become available for desumoylation of SIRT1.

In conclusion, I demonstrated that the modification of DBC1 by SUMO2/3 orchestrates with its ATM/ATR-mediated phosphorylation and SENP1-mediated removal of SUMO1 from SIRT1 for the control of the DBC1-SIRT1 interaction and in turn for p53-mediated apoptosis under genotoxic stress. Importantly, however, DBC1 has also been implicated in promotion of cell proliferation (Kim et al, 2009). For example, DBC1 binds to ER alpha, but its knockdown in MCF7 breast cancer cells enhances apoptosis in the absence of estrogen, suggesting the role of DBC1 as a positive regulator of cell growth (Trauernicht et al, 2007). Furthermore, it has been shown that the mRNA level of DBC1 is up-regulated in breast cancers, although not in prostate cancers (Fu et al, 2009; Radvanyi et al, 2005; Richardson et al, 2006). Thus, it appears that DBC1 plays a pleiotropic role in the control of cell growth and death, which might depend on cell- and tissue-types.

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국문 초록

포유류에서 SIRT1은 효모의 silent information regulator2 와 상동체로써, NAD^+-의존 히스톤 탈아세틸화 효소활성을 보인다. SIRT1은 세포사멸, 스트레스 반응, 암 생성 그리고 대사과정 등 세포 내 다양한 반응을 조절한다. 대표적인 암 억제 인자인 p53이 SIRT1의 주요 표적단백질이다. 스트레스가 가해지지 않은 환경에서는 p53은 SIRT1에 의해서 탈아세틸화되어 비활성상태로 존재하여, E3 유비퀴틴결합효소인 MDM2에 의해서 분해된다. 하지만 자외선이나 etoposide와 같은 스트레스가 가해진 환경에서는, p53은 p300/CBP 아세틸화효소에 의해 아세틸화되며, p53은 안정화되면서 활성화된다. 결국 활성화 및 안정화된 p53은 세포주기 중지나 세포사멸을 유도한다.

Small ubiquitin-related modifier (SUMO)는 유비퀴틴 유사 단백질로서 세포 내 여러 표적단백질들과 결합한다. 유비퀴틴화 시스템과 비슷하게, SUMO 또한 E1 활성 효소인 SAE1/SAE2와, E2 접합 효소인

Ubc9, 그리고 E3 결합효소인 PIASs들 효소에 의한 연속 전달 시스템으로 표적 단백질에 접합된다. 표적 단백질에 결합된 SUMO는 sentrin-specific proteases (SENPs)들에 의해서 반대로 제거될 수 있다. 이러한 가역적인 수모화 과정은 전사조절, 핵으로의 이동, 단백질 안정화 그리고 신호전달 과정 등 세포 내 다양한 프로세스를 조절한다.

Deleted in breast cancer 1 (DBC1) 은 이름에서도 알 수 있듯이, 암 억제인자로서 작용하며, 스트레스 반응이나 에너지 대사과정 등을 조절한다. DBC1은 앞서 언급한 SIRT1의 주요한 억제자이다. 세포에 스트레스가 가해진 환경에서는 DBC1은 SIRT1에 결합하여 SIRT1과 결합하고 있던 p53을 떼어내어 아세틸화를 유도하며 p53의 전사활성을 높여주어 하위 단계로 알려진 BAX, PUMA와 같은 세포사멸 유도 유전자들의 전사를 활성화하게 된다. 하지만 어떻게 DBC1이 SIRT1에 결합하여 조절되는지에 대해서는 밝혀진 것이 없다.

DBC1의 인산화는 DBC1과 SIRT1간의 결합에 영향을 주고 SIRT1의 탈아세틸화 기능을 조절한다. Etoposide와 산화 스트레스가 가해졌을 시,

ATM/ATR 인산화효소가 DBC1의 454번째 Thr기를 인산화한다. DBC1의 인산화는 SIRT1을 억제하고, p53의 아세틸화를 유도하여 p53에 의해 매개되는 세포사멸을 유도한다. 하지만 DBC1의 인산화 유사 돌연변이는 그 효과가 미미하다고 보고된 바 있다.

본 연구에서는 DBC1이 SUMO의 표적단백질임을 확인하고, 591번째 Lys기가 SUMO가 결합하는 위치임을 확인하였다. 또한 etoposide나 doxorubicin 처리에 의해 스트레스 상황이 유도되면 DBC1의 수모화가 유도됨을 보았으며, 이는 SUMO1이 아닌 SUMO2/3에 의한 것임을 밝혔다. DBC1의 수모화는 DBC1과 SIRT1간의 결합을 증가시켜주며, p53의 아세틸화를 유도하는 것도 확인하였다.

DBC1의 특이적인 SUMO E3결합효소는 PIAS3, 탈수모화효소는 SENP1임을 확인하였다. PIAS3와 SENP1은 DBC1 내의 같은 지역에 경쟁적으로 결합한다. Etoposide는 DBC1과 SENP1간의 결합을 감소시키고, PIAS3와의 결합을 증가시킴으로써 DBC1의 수모화를 증가시킨다. 앞선 실험 결과를 통해, ATM/ATR 인산화효소에 의한

DBC1의 인산화가 SENP1, PIAS3와 DBC1간의 결합에 상반된 영향을 줌으로써 DBC1의 수모화를 조절한다는 것을 확인하였다. 또한, SENP1 knockdown하였을 시, etoposide에 의한 세포사멸과정이 증가하고, 반대로 PIAS3와 SUMO2/3를 knockdown하거나, DBC1의 수모화가 유도되지 못하는 돌연변이를 과발현하였을 경우에는 세모사멸이 감소된다. 이 모든 결과를 통해, SUMO2/3에 의한 DBC1의 수모화가 p53에 의해 매개되는 세포사멸에 중요한 역할을 한다고 결론 내렸다.

Key word: DBC1(Deleted in breast cancer 1), p53, PIAS3 (protein inhibitor of activated STAT3), SENP1 (sentrin/sumo-specific protease 1), SUMO, phosphorylation, apoptosis

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